Separation and culture of cells isolated from the developing rodent cerebellum

Thesis

How to cite:

For guidance on citations see FAQs.

© 1980 The Author

https://creativecommons.org/licenses/by-nc-nd/4.0/

Version: Version of Record

Link(s) to article on publisher’s website:
http://dx.doi.org/doi:10.21954/ou.ro.0000fcad

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online’s data policy on reuse of materials please consult the policies page.
SEPARATION AND CULTURE OF CELLS ISOLATED
FROM THE DEVELOPING RODENT CEREBELLUM

D. NEIL CURRIE

A thesis presented to the Open University
in part fulfillment of the requirements
for the degree of Doctor of Philosophy.

Date of submission: 21.7.80
Date of award: 20.4.81
Frontispiece: "The experiments show that neuroblasts are competent to form primitive nerve fibers within a foreign unorganised medium simply by the amoeboid outgrowth of their protoplasm. By eliminating from the periphery all formed structures which have hitherto been supposed to transform themselves into nerve fibers and leaving only the neuroblasts in the field, it is demonstrated that the latter are the sole elements essential to the formation of nerves. The concepts of both Hensen and Held are rendered untenable."
PREFACE

This thesis describes a project which had a simple central objective: to take an existing preparation of dissociated cells from the immature cerebellum and develop conditions in which these cells could survive and differentiate in monolayer culture. The aim was to develop a system in which brain development could be investigated, particularly at the biochemical level in a simplified environment.

The study of cellular differentiation in vitro has two advantages:

1. Aspects of a cell's development which are intrinsically programmed in the cell at that stage can be distinguished from aspects which are dependent on some extrinsic influence, for instance from another cell type.

2. Cultures have a more restricted cellular composition than the brain and therefore may go some way to overcoming the cellular heterogeneity of brain tissue which renders interpretation of most conventional biochemical measurements difficult.

To realise the first it is necessary to compare a cell's differentiation in vitro with its normal course of development in vivo. To realise the second, cultures must be of a simplified and defined composition in which biochemical measurements can be attributed to particular cell types. Both requirements imply the use of cultures which contain in quantified proportions a few cell types which can be specifically
identified by reliable criteria, rather than in terms of such general categories as 'neurons'.

The use of a single area of the brain, the cerebellum, was an important first step in restricting the cellular composition of the cultures. The cerebellum contains large numbers of relatively few neuronal types which differ greatly in perikaryal size and therefore were amenable to a size-based separation of cell types by unit gravity sedimentation. Culture conditions were established for the survival and differentiation of the mixed cerebellar cell suspension and some of the separated cell fractions. This led to cultures containing a high proportion of granule neurons. However a fraction enriched in Purkinje cells did not survive well in culture. Finally, the composition of the cultures was further defined in a study involving cell type-specific immunological markers and the different uptake of the inhibitory neurotransmitter, GABA.

The description of this work is organised in seven more or less self-contained chapters, and can be informally divided into two parts. Chapter 1 is a general introduction to the problems of studying development, to the advantages of the cerebellum, in particular the detailed knowledge of some mechanisms of cerebellar development gained from work with mutants and X-irradiation and to the current knowledge, or lack of it, concerning the molecular basis of cellular interactions. Chapter 2 is a description of the cell isolation method used here, and of some improvements to the method effected during the course of this work. Chapter 3 completes the first part of the thesis and deals briefly with the history of brain cell separation techniques before
detailling the separation of cerebellar cells by a unit gravity sedimentation technique. This was a joint project with James Cohen and Gary Dutton at The Open University and Graham Wilkin and Robert Balazs at MRC, Carshalton. Chapter 3 concentrates mainly on the part of the project which was the primary work of this author, that is the size analysis of cells using a Coulter counter and points out those parts of the work, in particular electron microscopy, which were performed by others.

The second part begins with Chapter 4, which is a review of nervous system culture work with the aim of establishing whether there are reasons why the contribution of cell cultures to our biochemical knowledge has been quite limited, although they have been in use for many years and would appear an ideal controlled system for biochemical studies. Chapter 5 is a description of the search for culture conditions in which one week postnatal cerebellar cells would survive and grow, and of their pattern of development in culture. Chapter 6 details the use of markers to define culture composition more closely; cerebellar cultures were found to be over 80% in a single neuronal type, the granule cell. Cultures of embryonic cerebellum were found to contain a distinct population of neurons which are absent from cultures of older animals. Chapter 7 briefly summarises some future directions for this work and suggests that defined cell culture systems in alliance with modern immunological techniques, may offer the best route to find the elusive cell surface recognition molecules which are generally believed to mediate the cellular interactions that control development.
Some of this work, together with related aspects which are primarily the work of others, has been published elsewhere. Copies of these papers are attached.

ACKNOWLEDGEMENTS

My supervisor was Gary Dutton. He taught me many things, and always with friendship and humour. I am particularly grateful for his unfailing commitment to actually getting the science done, and done well, through all the obstacles presented by day to day work in a laboratory and research group. I have worked closely with Jim Cohen, Kathleen Tear, Richard Beale, Malcolm East, Rod Pigott and Brian Pearce, it was all unreservedly a pleasure. I would also like to thank Steven Rose and other members of the Open University Biology Department, past and present, for the many occasions on which they have assisted me. Several colleagues from other laboratories have supplied me with essential materials; they are mentioned in the text, and I thank them. I gratefully acknowledge Hoechst Pharmaceuticals (U.K.) Ltd and the Medical Research Council who have provided grants in support of this work. Mary Golden typed this thesis with her customary excellence, and patience in confronting my writing.
## CONTENTS

### Chapter

<table>
<thead>
<tr>
<th>1 General Introduction</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Preparation of Cerebellar Cell Suspension</td>
<td>23</td>
</tr>
<tr>
<td><strong>Methods</strong></td>
<td>26</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>30</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td>35</td>
</tr>
<tr>
<td>3 Cell Separation</td>
<td></td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>39</td>
</tr>
<tr>
<td><strong>Methods</strong></td>
<td>49</td>
</tr>
<tr>
<td><strong>Calculation of Coulter Results</strong></td>
<td>58</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>65</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td>93</td>
</tr>
<tr>
<td>4 Introduction to Cell Culture</td>
<td>100</td>
</tr>
<tr>
<td><strong>Explant Cultures</strong></td>
<td>102</td>
</tr>
<tr>
<td><strong>Re-aggregating Cultures</strong></td>
<td>109</td>
</tr>
<tr>
<td><strong>Monolayer Culture of the Brain</strong></td>
<td>127</td>
</tr>
<tr>
<td><strong>Continuous Cell Lines</strong></td>
<td>146</td>
</tr>
<tr>
<td><strong>Culture Models</strong></td>
<td>149</td>
</tr>
<tr>
<td>5 Cell Culture of Cerebellum</td>
<td>153</td>
</tr>
<tr>
<td><strong>Methods</strong></td>
<td>154</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>157</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td>189</td>
</tr>
<tr>
<td>6 Cell Identification in Culture</td>
<td></td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>196</td>
</tr>
<tr>
<td><strong>Methods</strong></td>
<td>201</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>206</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td>231</td>
</tr>
<tr>
<td>7 Future Directions</td>
<td>235</td>
</tr>
</tbody>
</table>

**References** 239
Chapter 1

GENERAL INTRODUCTION

The purpose of an introduction is to set a context for the ideas which follow. It is therefore appropriate to dwell briefly on some broader issues of developmental neurobiology before narrowing to a description of a quite specialised approach to a particular problem. More detailed literature reviews of the specific topics of brain cell separation methods and culture of the nervous system are included in later chapters.

The primary interest of neurobiology is the human brain, mainly because it is our own, but also because it appears to be the newest and most "advanced" in evolution, capable of performing the most complex integrative tasks. The human brain contains more than $10^{10}$ neurons each of which connects to thousands of others (each cerebellar Purkinje neuron receives input from $10^5$ other neurons). The meaning
of such a system is beyond our present imagination. Neurobiologists are attempting to answer two questions about the brain: how does it work, and how is it formed during development? At some level these two questions become one, for it is clear that not only does the brain's structure underline its function, but also that its function must modify that structure. It is difficult to over-estimate the plasticity of the nervous system, particularly of mammals and particularly during development.

The mechanism by which the brain performs the functions that we know from subjective experience and from psychology remains totally obscure. We shall probably remain very distant from a neurobiological explanation for a long time. The means by which such a complex system constructs itself from a single cell using a quite limited number of genes is almost as obscure. The details of structure may be modified by environmental factors of a minute (memory) or extreme (under-nutrition) nature. However it is clear that the basic structure of the brain and its ability to respond to environment results from a sequence of interdependent events which originate in genetic programming.

The work detailed in the succeeding chapters is addressed to the question of brain development. A section of the cerebellum stained by the Golgi technique looks highly complex and at first glance without order (Figure 1.1a); yet, when specific cell types are abstracted from the same drawing, it becomes clear that dendritic arborisation within a specific neuron class is remarkably "typical"
The intricate nets of interacting neuronal processes which form so complex a pattern are fascinating simply for their aesthetic quality. However even more fascinating, from the biological standpoint, is the regularity with which this network is reproduced from individual to individual and to a lesser extent through evolution. How do the cells of the brain move to their correct positions during development, form specific connections and finally establish their complex and yet reproducible inter-relationships? What are the 'forces' which form the shapes? (Roux, 1895 quoted by Rakic, 1974).

It has been estimated that there are more types of cell in the brain of a mammal than in all the rest of the body (Rakic, 1974). There is considerable biochemical diversity among brain cells, but the most notable element of complexity is shape. Most neuron types have been identified on the basis of shape, initially by the classical anatomists using the Golgi stain. Differences in their physiological properties which have since emerged can be regarded as due firstly to the differences in anatomy including microanatomy and secondly to differences in transmitters, receptors, ion gating etc. which can be understood, or at least conceptualised, at a biochemical level. Such biochemical differences between neurons must be due to qualitative and quantitative variations in proteins i.e. to differential expression of genes under a control mechanism which is not understood, but which need be no different from that which operates to control all differentiation between cells in a multi-cellular organism.
Fig. 1: A. Composite drawing of the structural pattern of the mature cerebellar cortex in rhesus monkey, as seen in the plane transverse to the folium in the anterior lobe close to the midline. All cellular images were drawn from 125 μm thick rapid Golgi preparations at magnification of 490 diameters with the aid of Zeiss drawing tube and then reduced photographically for publication. The scale at the bottom indicates 100 μm. The modified rapid Golgi method used for impregnation in this study stains neurons and glia equally well. All cells, except blood vessels, are included in this drawing. Abbreviations: GL, granular layer; ML, molecular layer; PL, Purkinje cell layer. B. A few neuron images are abstracted from the composite drawing in A to illustrate typical shapes of Purkinje cells (P), granule cell (G), interneurons of the molecular layer (I), and Golgi type II neuron (GII). (from Rakic, 1974)
However the morphological complexity of the brain poses a special problem. It has been often calculated that the number of specifications required to build and operate a complex brain is too great for the mammalian genome (e.g. Bremermann, 1963). Therefore, it becomes necessary to postulate (e.g. Jacobson, 1970) that the shapes of only a small proportion of neurons may be intrinsically specified while the remainder develop their shape in response to their local environment; that is they only require instructions to 'read' the 'signposts' produced by other cells. It is proposed that there are a few 'primary' events, i.e. aspects of a cell's development which are intrinsic to that cell type and not dependent on interactions with other cells. These are followed by many 'secondary' events which depend on the primary cues. The origin of a cell type itself must of course have depended on interactions between cells, but this distinction between primary and secondary events, although artificial, is a useful construct which clarifies investigation of the causal relationships between developmental events.

Much is known from the classical work of embryologists and neuro-anatomists of the cell movements and general structural changes during the development of the nervous system. However it is quite impossible to perceive the mechanisms, the causal relationships, behind such changes in an interdependent system unless the system can be perturbed in a controlled manner. Some experimental perturbations of development are described below. The approach of the work in the following chapters is to develop a system in which some aspects of brain development can be studied in a more limited
context. Many of the variables present in the brain can then be eliminated, or preferably controlled at will.

Three simplifying assumptions or restrictions have been made in this work. These are (a) that rodents are a good model for humans, (b) a limitation to one area of the brain, the cerebellum, and one stage of development, and (c) a further sub-division of the cerebellum to study the individual cell types as they develop in culture.

The use of rodents as a model for humans has proven an excellent assumption for the study of basic metabolic processes. Indeed, at this level bacteria are often an acceptable approximation. It is a very doubtful assumption, though one often made, for the study of complex behaviours involving the aspects of the brain in which rodents and humans are least similar. The justification for the use of this model in studying basic mechanisms of development in the brain rests in part on the wealth of detailed anatomical studies which show great similarities in the overall cell movements in developing brains of the rat, cat, monkey and human.

The Cerebellum

The highly regular cortical array of the adult cerebellum is a structure of great beauty which is conserved in broadly similar form throughout the evolution of the vertebrates. From the earliest days of neuroanatomy there has been interest not only in the adult structure, but also in the ordered sequence of events by which it
develops. The first period of great anatomical interest in cerebellar development began with the identification of the different cellular and laminar constituents of the developing cerebellar cortex by workers of the French and German schools in the last two decades of the nineteenth century (see Altman, 1972a). This period culminated in an extended series of Golgi studies by Ramon y Cajal (1960) who gradually succeeded in unravelling the major steps in the migrations and transformations of the different cell types. He did not hesitate to infer functional interactions from the anatomical interconnections which he saw in the adult cerebellum. However his scheme for the cerebellum shown in Figure 1.2 is limited by a lack of knowledge of inhibitory synaptic action, although the directions of nerve impulse flow were accurately described. The importance of postsynaptic inhibition in the cerebellum was revealed during the succeeding decades of physiological work until it was possible for Eccles, Ito and Szentagotthai (1967) to attempt, with some success, a synthesis from the physiological and anatomical data of a complete description of the cerebellum as a "neuronal machine". This work included anatomical information revealed by electron microscopy, the first important new neuroanatomical technique since the Golgi procedure and with the work of Palay and Chan-Palay (1974) the cerebellum is now the area of the brain which has been most completely described at this ultrastructural level.

The structure of the adult cerebellum has been described by several recent authors, accompanied by elegant diagrams (Eccles et al., 1967; Rakic, 1973; Palay and Chan-Palay, 1974). Those shown in
Fig. 1.2: Two diagrams of Cajal (1911, Figs. 103 and 104) showing that, although not fully appreciating the significance in this contact of the dendritic spines, he correctly understood the principle of the "crossing over" synaptic system between the unmyelinated parallel fibers and the various kinds of dendrites in the molecular layer. Diagram at left shows arrangement with Golgi cell (c) and at right with basket cell (b). Legends in left diagram: A = mossy fiber, a = granule neuron, b = parallel fiber, c = Golgi cell, d = Purkinje cells, B = Purkinje axons. Right diagram: A = mossy fiber, a = granule neuron, b = basket cell, B = Purkinje axon, c = Purkinje cells, d = climbing fibers. Arrows indicate direction of impulse flow. (from Eccles et al., 1967)
Figures 1.3 and 1.4 are taken from a particularly clear statement of the basic organisation by Llinas in Scientific American (1975). For the details of synaptic interaction it is best to refer to Palay and Chan-Palay (1974).

Electron microscopy has also advanced the description of cerebellar development giving a more detailed view of cellular ultrastructure and providing information on the dynamics of synapse formation. A second new technique of importance is the labelling of dividing cells in vivo by injection of $^3$H-thymidine and the detection of their subsequent fate by autoradiography of tissue sections. This permits conclusive determination of the sequence of origin of different cell populations and of their migration patterns. Extensive recent studies of development have been made by Sidman (1974), Rakic (1974), Sotelo (1975) and a series of papers by Altman (1972a, b, c; 1973a, b; 1975; Altman and Anderson 1972, 1973).

These workers and others have documented in great detail the course of normal development using electron microscopy and $^3$H-Thymidine autoradiography in addition to classical light microscopic histology. They have also attempted to establish causal relationships between the interdependent events of development by studying the effects of specific perturbations of its normal course. The cerebellum is a particularly favourable area for such studies for two unrelated reasons. Firstly, because of its highly ordered sequence of neurogenesis it is possible by carefully timed cytotoxic interventions
ARCHITECTURE OF THE CORTEX of the cerebellum is diagrammed for a section of tissue from the brain of a cat. The location of the tissue section is indicated in the drawing at top right; the same array of cells is repeated throughout the cortex. Each cell type is identified by color in the key at bottom left. The cortex is organized around the Purkinje cells, whose somas, or cell bodies, define the border between the superficial molecular layer and the deeper granule cell layer. In the molecular layer are the Purkinje cell dendrites, which are arrayed in flattened networks like pressed leaves, and the parallel fibers, which pass through the dendrites perpendicularly. This layer also contains the stellate cells and the basket cells, which have similarly flattened arrays of dendrites. In the deeper layer are the granule cells, which give rise to the parallel fibers, and the Golgi cells, which are characterized by a cylindrical dendritic array. Input to the cortex is through the climbing fibers and mossy fibers, output is through the axons of Purkinje cells.

Figure 1.3

(from Llinas, 1975)
Figure 1.4: INTERCONNECTION OF NEURONS in the cortex follows an elaborate but stereotyped pattern. Each Purkinje cell is associated with a single climbing fibre and forms many synaptic junctions with it. The climbing fibre also branches to the basket cells and Golgi cells. Mossy fibres come in contact with the terminal "claws" of granule-cell dendrites in a structure called a cerebellar glomerulus. The axons of the granule cells ascend to the molecular layer, where they bifurcate to form parallel fibres. Each parallel fibre comes in contact with many Purkinje cells, but usually it forms only one synapse with each cell. The stellate cells connect the parallel fibres with the dendrites of the Purkinje cell, the basket cells mainly with the Purkinje-cell soma. Most Golgi-cell dendrites form junctions with the parallel fibres but some join the mossy fibres. Golgi cell axons terminate at the cerebellar glomeruli. Cells are identified in the key at lower left; arrows indicate direction of nerve conduction. (from Llinas, 1975).
such as X-irradiation or chemical treatments to damage or destroy specific populations of cells. Secondly, the cerebellum is involved in motor coordination and therefore deficiencies in cerebellar function are particularly conspicuous in the behaviour of the affected animal. This has led to the recognition of several spontaneously occurring neurological mutants of the mouse whose names attest to the properties which allowed their detection e.g. reeler, weaver, staggerer and nervous. There are now seven such single-gene neurological mutations which affect the structure and function of the cerebellum and which have been established on inbred backgrounds, generally at the Jackson Laboratory in Bar Harbor, Maine (reviewed by Caviness and Rakic, 1978).

The cerebellum is thus well suited to provide answers to questions concerning the interplay of intrinsic and extrinsic factors influencing neuronal development. A full description of cerebellar development as it is currently understood will not be attempted here. However a few salient points will be discussed with the aim of illustrating the level of developmental analysis which is now possible in anatomical studies of this system as a preamble to a consideration of the possibilities of in vitro experimentation.

The first cerebellar cortical neurons to be generated are Purkinje cells which arise on embryonic day 15-16 in the rat (Das and Nornes, 1972). Their dendritic growth passes through a multipolar stage before the development of typical primary, secondary and tertiary branching leading to the final flattened dendritic
tree (Rakic, 1971). As the dendrites grow they become studded
with numerous spines which are normally the post-synaptic site for
innervation by parallel fibres (granule cell axons). Since Purkinje
cells are the first neurons to develop, it might be expected that the
initial formation of their dendritic tree was under autonomous
control.

The first indication that Purkinje dendrites develop independently
of their main afferent input, parallel fibres, came from Golgi studies
on reeler and weaver mutant mice (Sidman, 1968). The primary branching
and spine development of the Purkinje dendritic tree was maintained in
cells growing in the absence of parallel fibres. This remained true
if the granule cell loss was due to X-irradiation (e.g. Altman and
Anderson, 1972). However tertiary branches were poorly developed,
and orientation of the dendritic tree appeared completely random.
It is remarkable that Purkinje dendritic spines, and even the
post-synaptic specialisations which they bear, develop in weaver mouse
in the absence of parallel fibres, their pre-synaptic input (Rakic
and Sidman, 1973b, c) and remain stable for the life of the animal,
thus this part of one synapse type appears to be programmed
independently of contact between pre- and post-synaptic elements.

The conclusion that the primary branching of the Purkinje
dendritic tree and spine formation is intrinsically programmed to
that cell must be qualified, as other influences may be in force.
In particular, climbing fibres form early contacts with Purkinje
cells, as do catecholaminergic axons from the locus coeruleus. However spines continue to form on Purkinje cells growing in explanted cerebellum which is devoid of fibres of external origin (Privat, 1975). There is strong evidence that later stages of Purkinje arborisation do rely on interactions with parallel fibres. In experiments where the growth of granule cell axons was disturbed, but not prevented, by limited irradiation it was found that in areas where the orientation of parallel fibres was altered by 90° then the Purkinje dendritic tree was similarly re-oriented to remain at right angles to the parallel fibre array (Altman, 1973b). Parts of the dendritic tree of the same cell could be mutually perpendicular, implying that the tertiary growth of Purkinje dendrites is under the control of influences in their immediate locality. A quantitative analysis of the growth pattern of Purkinje dendrites under agranular conditions (Berry and Bradley, 1976) favoured the interpretation that the degree of dendritic branching depends on the number of synaptic sites available during the period of growth, in accordance with the hypothesis (Vaughn et al., 1974) that transient randomly occurring branchings of the growth cone are 'fixed' by the formation of synapses.

It appears, therefore, that Purkinje dendrites including spines can be elaborated and maintained in the absence of their major input, parallel fibres, although interaction with parallel fibres does affect their detailed tertiary development. It may then be asked whether the converse holds true, whether parallel fibres can be elaborated
and maintained in the absence of their postsynaptic element. Fortunately the staggerer mutant can cast some light on this question. Growth of the Purkinje dendritic tree is stunted in staggerer cerebellum, and there are no dendritic spines (Sidman, 1972). Granule cells proliferate and migrate inwards, passing through their normal stages (Rakic, 1971) and generating an array of parallel fibres which then fail to form synapses with the abnormal Purkinje dendrites. Subsequently virtually all granule cells die, and this suggests that granule cells, unlike Purkinje cells, are dependent upon the formation of the parallel fibre/Purkinje dendrite synapse (Sidman, 1972).

There has been some controversy over the mechanism of granule cell migration. 'Glial guidance of the migrating granule cells was proposed on the basis of electron microscopic studies which showed descending granule cells in apposition to the ascending fibres of Bergmann glial cells. The apparent abnormality of Bergmann fibres in the weaver cerebellum, where granule cells are generated but then fail to migrate and die, was cited as strong evidence that "the Bergmann glial disorder precedes the migration defect temporally and causally" (Rakic and Sidman, 1973a). However other workers have suggested that Bergmann glia arise too late to fulfil this purpose (Das et al, 1974) and that, when visualised by immunofluorescence with an antibody to glial fibrillary acidic protein, the Bergmann fibres are initially tortuous and do not become radially aligned until two weeks after birth (Bignami and Dahl, 1973) at which time there are many normal fibres in the weaver cerebellum. Altman (1975) has argued from this evidence, and from
his own work on re-orientation of the parallel fibre array by X-irradiation, that the growth pattern of Bergmann fibres depends on the organisation of granule cell processes rather than vice versa.

The two classes of inhibitory interneuron present in the molecular layer, stellate and basket cells develop their axonal outgrowth at right angles to the parallel fibre array. This results in the earliest-arising cells being 'fixed' in a low position in the molecular layer, and the last being fixed in an upper part, as the parallel fibres are laid down from above (Altman, 1972a). Rakic (1972) has argued from an extensive study of cerebellar development in monkeys that the outgrowth of stellate and basket cell dendrites is dependent on the number of parallel fibre pre-synaptic sites available. Thus the early-arising deeply-placed cells extend dendrites upwards throughout the molecular layer, but the superficially-placed cells have a more restricted field of growth downwards, perhaps because of the limited number of sites remaining vacant as their dendrites elaborate. In areas of weaver mutant cerebellum which are devoid of parallel fibres, the molecular layer interneurons survive but with reduced and disorganised dendritic trees (Rakic and Sidman, 1973b), thus corroborating a dependence of stellate/basket dendritic outgrowth on the parallel fibre array.

These examples illustrate a range of dependence of CNS neurons on extrinsic influences and how those dependencies can begin to be delineated, although not yet determined, by studies of the cerebellum taking advantage of its ordered development and the available
experimental and genetic manipulations. Thus, Purkinje cells appear to develop relatively autonomously with respect to basic structural properties of branching pattern and spines while their final dendritic arborisation depends on parallel fibre interactions. However, the role of climbing fibres and other early afferents cannot be discounted (Hamori, 1973). Granule cells may, in their formation of parallel fibres, provide the primary influence on cerebellar cortical orientation (Altman, 1975) or this may be an attribute of other cells (Rakic and Sidman, 1973a). However granule cells depend for their survival on forming the correct synaptic interactions. The dendritic development of stellate and basket cells exhibits a considerable and reproducible diversity which can be completely accounted for by influences extrinsic to those cells.

The clear logic behind all attempts to distinguish intrinsic and extrinsic influences on neuronal development is the proposition that if a cell is dependent on an extrinsic influence, manipulation of that influence will change the cell and, conversely, that if manipulation or removal of an influence does not change the cell (or one of its properties), then the cell (or property) is independent of that particular influence. Mutants, X-irradiation, and other experimental procedures provide means to manipulate such influences. Further experimental possibilities arise if growth is studied in cultured explants of cerebellum, where, for instance, there are no climbing fibres and mossy fibres present (Seil, 1979; and review in Chapter 4).

A more radical approach would be to isolate a cerebellar cell
(or cell type) from all normal extrinsic influences in purified culture. Those normal in vivo properties of the cell which were still reproduced under such conditions could then be strongly argued to be truly intrinsic to the cell. (That is, intrinsic to the cell at the age isolated; all cellular differentiation from the fertilised egg is likely to be dependent on cellular interaction at some stage). All properties not reproduced are probably dependent on some extrinsic influence. This can be tested by 'adding back' normal in vivo influences (such as the presence of another cell type) and looking for the restoration of a missing property. The potential importance of defined and purified culture systems, and the experimental pre-requisites for obtaining them are discussed in detail in Chapter 4, they have not yet been fulfilled in any CNS system.

The Nature of Cellular Interactions during Development

The description so far, and most of the work referred to has depended on terms such as 'extrinsic influence' and 'cellular interaction' to name phenomena whose presence can be inferred from developmental studies. A biochemist must ask: what is the nature and location of the molecules which mediate this influence of one cell on another? Are there special 'recognition' molecules, and if there were, how would one find them?

It has long been proposed that neuronal connections are established on the basis of specific biochemical cues; this idea has
its most well-known exposition in the 'chemoaffinity hypothesis' (Sperry, 1963). Alternative explanations have mainly involved timed outgrowth of fibres, the idea that fibres arrive at a target region and occupy sites in a specified order, thus avoiding the need for direct, specific affinity between fibre and site. Such mechanisms may play a part, but there are now many results from developmental experiments which can only be accounted for on the basis of some more specific recognition (Gottlieb and Glaser, 1980). Most of the work in this area has come from developmental experiments, often manipulations of the connections between retina and tectum in amphibians and birds. The results of such experiments now argue quite strongly for some chemically based recognition between neurons (for reviews: Gottlieb and Glaser, 1980; Fraser and Hunt, 1980). The next step is clearly to move to biochemical experiments, to searching for molecules which can be shown to mediate recognition between cells.

It is not possible for there to be 'one molecule, one synapse' or even 'one molecule, one neuron'; the mammalian genome is not large enough to carry such information, however it is hard to see how the specific events of development can take place without a repertoire of specific molecules by which cells can recognise each other. Such molecules could have other functions, for instance different local synaptic sites for an incoming fibre could be distinguished on the basis that only the correct site carries the receptor for the transmitter to be released by that fibre. However the establishment of a retino-tectal map cannot be explained by such simple cues, and must depend on means to distinguish between neurons of the same type. It has been proposed that the great
specificity observed does not require a multitude of different molecules, but could be explained by quantitative differences between cells in expression of a recognition molecule, or in the ratio of two such molecules, thus establishing gradients across the tectum or other target area (e.g. Fraser and Hunt, 1980). Clearly such explanations will remain highly conjectural until the molecules involved can be recognised, studied, and quantified. Cell-surface glycoproteins are strong candidates as recognition molecules, and biochemical mechanisms by which such recognition could occur have been proposed (e.g. Barondes, 1970; Edwards, 1978).

The limitation remains, however, that few, if any, 'recognition molecules' have yet been found. The reasons for this are quite clear; there are no reliable assays for such molecules, therefore no-one can know if they find one. Furthermore most standard biochemical techniques apply only to large numbers of cells, or components extracted from them. Currently this means that, within the nervous system, cellular recognition can only be studied in very heterogeneous collections of cells. This cannot be reconciled with the developmental studies that suggest precise specification of cell interaction, and allow distinctions to be made between cells in adjacent regions.

One approach is to study adhesiveness between cells *in vitro* in the expectation that this is related to cellular recognition *in vivo*. There is much evidence that brain cells adhere preferentially to other cells from their own brain region, and even that retinal cells adhere
preferentially to cells from the area of tectum with which they
would normally interact (Barbera, 1975). An antiserum to a cell
surface molecule was found to interfere with specific adhesion in
such an 'assay', implicating the target antigen in the adhesion
phenomenon (Rutishauser et al, 1979). This molecule, designated
CAM (cell adhesion molecule) is widely distributed in the nervous
system and may be a common component of the cell adhesion mechanism.
Anti-CAM antiserum prevents the formation of neurite bundles in
dorsal root ganglion cultures, but the involvement of CAM in cellular
recognition in vivo has yet to be demonstrated. This one rather
generally distributed protein is the only established candidate for
a molecule involved in cellular recognition.

There are thus two problems obstructing progress in this field.
Firstly, a general problem for the neurochemist, the brain is by
definition heterogeneous, it deals in information and therefore,
unlike the liver, every cell (neuron) must be in some respect
different from every other. Even carefully dissected specific brain
areas will contain many different cell types and the differences within
a particular cell type, as in the retinotectal system, may be
highly significant for development (and of course for function).
The continuing quest for the biochemist must be to develop experimental
systems which are more homogeneous, without losing expression of the
particular function which is under study. These issues are discussed
further in Chapter 4. The second problem in looking for the
molecular basis of cellular recognition is that there are few, if
any, methods of assaying for such molecules. It is difficult for
a biochemist to work with proteins which have no assayable property such as enzyme activity. One exception is the nicotinic acetylcholine receptor, a non-enzyme membrane protein which has been isolated and extensively characterised, but only because there was a specific tight-binding ligand available, α-bungarotoxin. The methods of modern immunology probably offer the best possibility to generate a range of specific ligands against unknown proteins. However such methods require fairly homogeneous experimental systems for assay of antibodies.

The work described in this thesis is addressed to developing a system in which one well-understood area of the brain can be studied as its component cell types in culture. Their in vitro development can then be monitored, in order to distinguish intrinsic and extrinsic factors in the control of development, and a more homogeneous system is provided for studies of cellular recognition.
Dissociation of the complex structure of the brain into its component cells is a rather drastic procedure which has been used by many investigators in attempts to resolve the heterogeneity of brain tissue. Most methods include the mechanical disruption of the tissue by sieving through fine nylon or steel meshes or by repeated pipetting (trituration). Sometimes this is augmented by the use of digestive enzymes, most commonly trypsin, or other chemical agents such as EDTA.

Cell dissociation methods can be divided into two broad groups. There are those which have been devised primarily by biochemists to produce a bulk preparation of cells (cell perikarya) to which fractionation procedures, usually gradient centrifugation can be applied. These are briefly reviewed in the introduction to Chapter 3 on cell separation methods. They generally involve the use of fairly mature tissue and lead to preparations which have been extensively characterised in metabolic studies. Yields are often low (e.g. 6% - Rose, 1967; 5.6% - Sellinger et al, 1971) and there has been some controversy over the quality of ultrastructural
preservation at the EM level. These cells certainly do not survive well in culture.

The second group of methods has a different origin, the necessity to dissociate tissue for the production of primary monolayer or aggregating cultures. Similar techniques of mechanical or mechanical/ enzymatic disruption have been used, but in this case on immature or embryonic tissue. The main criterion for success of such methods has been the production of cultures which will show long-term survival of neuronal cells. There has therefore been little interest in the metabolic or ultrastructural preservation of the cells before plating, or in the overall yield of cells from the tissue. Primary culture work is reviewed in Chapter 4.

Drs. James Cohen and Gary Dutton at the Open University, developed a method for the preparation of cells from the postnatally developing rat cerebellum (Cohen et al, 1974; Wilkin et al, 1976). This procedure combined some of the advantages of each group of methods mentioned above. The main difference in their approach was to take high overall yield and good ultrastructural preservation as the primary criteria for assessment of the preparation. Electron microscopic monitoring was used to find the conditions which gave consistently good ultrastructural preservation in low power surveys of large numbers of cells. (EM on cell preparations was performed in collaboration with Drs. Wilkin and Balazs at the MRC Developmental Neurobiology Unit, then in Carshalton, and now in Queen Square, WCl.) Cohen's controlled trypsination procedure which is described in detail below, was a broadly conventional
mechanical/enzymatic procedure. However it differed in that the concentration of trypsin found to be optimal was very low (0.025%, 40-fold less than used in several contemporary bulk isolation and culture preparation methods, e.g. Norton and Poduslo, 1970), the trypsin reaction was stopped using soya bean trypsin inhibitor and all solutions were based on a bicarbonate buffer supplemented with glucose and bovine serum albumen.

Phase contrast microscopy of cell suspensions prepared by this method showed large numbers of small (<10μm) spherical cells and about 1-2% of larger (~20μm) spherical cells. Electron microscopy revealed that the most common cell type was a small electron dense cell with a thin rim of cytoplasm surrounding an invaginated nucleus containing large blocks of condensed chromatin. This is similar to external granule neuroblasts seen in situ in sections, although at this age it is not easy to distinguish these cells from granule neurons at later stages of their differentiation without the positional cues given by in situ observation. The majority of the large cells could be tentatively identified as Purkinje neurons. They displayed an undisrupted plasma membrane, cytoplasmic organelles, and a characteristic asymmetrically placed nucleus with nuclear folds present in the direction of the mitochondria-rich pole of the cell. The cells were metabolically competent by several criteria including coupled respiration, accumulation of K⁺ against a concentration gradient, and incorporation of labelled amino acid precursor into acid insoluble material. Amino acid
incorporation was linear over three hours and was inhibited by cycloheximide. The cell preparation method described in this Chapter has been amended to incorporate sterile precautions so that the resulting cells may be cultured.

During the course of the work described in succeeding chapters this technique was re-evaluated and a modification of the 'original' method was developed with the twin objectives of further improving the properties of the cells and of simplifying and shortening the procedure. The results described in this Chapter are of a comparison of the modified method with the original method in terms of cell yield, cell size distribution and ultrastructural preservation.

**METHODS**

Wistar (CFHB) rats 2-14 days of age were used. The date of birth was recorded as day one with an associated margin of error of 12 hours. Litters of ten or more pups were randomly divided, and animals from each half used for either the original or modified cell isolation method. All operations were performed at room temperature unless otherwise stated. All media were gassed with a mixture of 95%\textsubscript{O}_2/5%\textsubscript{CO}_2 to pH 7.4 before use, and glassware was siliconized with Repelcoote (Hopkins and Williams Ltd., U.K.).

For sterile preparations, all media were pre-filtered by vacuum through a 0.45\textmu m pore size Millipore filter, enzyme solutions were
then made up, and all solutions were finally dispensed using a syringe and sterile 3cm Swinnex filter holder containing a 0.22μm pore size Millipore filter. Siliconised glassware was sterilised by dry heat at 160 °C, pasteur pipettes being plugged with cotton wool. Plastic and rubber items such as pipette teats were sterilised with 70% ethanol. New, sterile 10ml plastic conical tubes were used where required. All manipulations were performed in a laminar flow hood (Microflow, U.K.), tubes were capped before transfer to the centrifuge.

The first few steps were common to both techniques and were performed as follows: animals were swabbed with alcohol, decapitated and cerebella removed, pooled and cleaned of meninges. Pooled cerebella, equivalent to 150 - 200 mg, wet weight of tissue were chopped at 400μm intervals with two passes (the second pass 90° to the first) using a McIlwain tissue chopper (Mickle Labs, U.K.). At this point the remaining steps in the two procedures diverged as described below.

**Original method**

All media were based on a Krebs-Ringer bicarbonate (KRB) solution containing 14mM glucose and bovine serum albumin (BSA, fraction V, Sigma) (KRB/BSA). The BSA concentration was increased from 3 mg/ml for tissue blocks to 5 mg/ml for dissociated cells. KRB contains: 118mM NaCl, 25mM NaHCO₃, 4.8mM KCl, 2.5mM CaCl₂, 1.2mM KH₂PO₄, 1.2mM MgSO₄.
After chopping, tissue blocks were gently dispersed in 15 ml KRB/BSA, and sedimented at approximately 50g for 5 sec. in a 50 ml conical glass centrifuge tube. The supernatant was decanted and the pelleted material resuspended in 10 ml KRB/BSA containing 0.25 mg/ml (0.025%) bovine pancreatic trypsin (type III, twice crystallized, Sigma) and rapidly transferred to a 35 ml trypsinization flask which was capped and incubated in a shaking water bath for 15 min. at 37 °C. Incubation was terminated by the addition of 10 ml KRB/BSA containing 4 μg/ml DNAse I (Sigma) and the flask contents returned to the centrifuge tube. DNAse action was allowed to continue while the tissue blocks fell freely to the bottom of the tube (about 1 min.).

After brief centrifugation as above, the sedimented tissue was resuspended in 10 ml KRB/BSA containing 0.25 mg/ml chromatographically pure soya bean trypsin inhibitor (SBTI, Sigma), and incubated for 5 min. at 37 °C. The tissue blocks were again pelleted as above, resuspended in 10 ml. Ca²⁺ and Mg²⁺-free KRB/BSA (CMF-KRB/BSA) containing 2mM EDTA, and incubated for a further 5 min. at 37 °C. The sedimented tissue was washed 3 times with 20 ml CMF-KRB/BSA, each followed by centrifugation and decantation as described above.

Finally, the softened tissue blocks were resuspended in approximately 2 ml of Ca²⁺-free KRB/BSA containing 40 μg/ml DNAse I and triturated using a siliconized Pasteur pipette. The use of a high concentration of DNAse at this point was essential to prevent clumping caused by released chromatin. After trituration 25 times (up and down passes) the undisrupted material was allowed to settle (approximately 5 min.). The cloudy supernatant containing the isolated perikarya was transferred using a pasteur pipette to a 10 ml conical plastic centrifuge
tube containing 2 ml of KRB/BSA (0.1 mM Ca\(^{2+}\)). The undisrupted tissue was again triturated as above and the supernatants pooled. Finally, the perikarya were separated from most of the small debris by underlying the supernatant with 2 ml of 40 mg/ml BSA in CMF-KRB and centrifuging for 5 min. at 100xg. The pelleted cells were carefully resuspended with a siliconized Pasteur pipette in a medium appropriate for their subsequent use (see following chapters).

**Modified method**

All media were based on Ca\(^{2+}\) and Mg\(^{2+}\)-free Earle's Basic Salt Solution (Gibco-Biocult Ltd.) supplemented with 3 mg/ml BSA (fraction V, Sigma), 14mM glucose and 1.5 mM MgSO\(_4\) (CF-EBSS/BSA). Chopped cerebella were dispersed in 10 ml of CF-EBSS/BSA containing 0.25 mg/ml (0.025%) trypsin (type III, Sigma), transferred to a 50 ml trypsinization flask which was capped and incubated for 15 min. at 37 °C in a shaking water bath. Incubation was terminated by the addition of 10 ml of CF-EBSS/BSA containing 6.4 μg/ml DNase I (Sigma) and 80 μg/ml soya bean trypsin inhibitor (SBTI, Sigma). The flask contents were transferred to a 50 ml glass conical centrifuge tube and centrifuged at 50g for 5 sec. The supernatant was discarded and the tissue blocks resuspended in 2 ml of trituration solution - CF-EBSS/BSA containing 40 μg/ml DNase I, 0.5 mg/ml SBTI and MgSO\(_4\) increased to 3mM. The material was triturated 12-15 times using a siliconized Pasteur pipette and the undisrupted tissue allowed to settle (3-5 min.). The supernatant was transferred to a 10 ml conical plastic centrifuge tube, the trituration procedure was repeated on the undisrupted material and the supernatants combined.
Finally, the perikarya were separated from most of the small debris by underlying the supernatant with 2 ml of 40 mg/ml BSA in CF-EBSS/BSA and centrifuging for 5 min, at 100g. Pelleted cells were resuspended using a siliconized Pasteur pipette in a medium appropriate for their subsequent use.

Cells were counted using a Coulter Counter, Model ZB (Coulter Electronics Ltd., Harpenden, U.K.) with a 140μm orifice tube. Counts of cells in particular size ranges were made by adjusting the upper and lower threshold on this machine, which had been calibrated using polystyrene beads of a known size. The theory of the Coulter Counter is described in the following Chapter. DNA content of whole cerebellar tissue and of perikaryal preparations was determined by method of Zamenhof et al (1964).

RESULTS

The much shorter modified procedure is the result of several changes in the original protocol. Ca++-free solutions are used throughout and the EDTA incubation step is eliminated, as are the following three washes. The separate incubation with trypsin inhibitor is dispensed with and SBTI is added to the DNase solution. The number of solutions required is reduced from seven to three, all of which use the same Earle's salts solution which is the basis of many culture media. The time required for cell isolation is reduced from 90-120 minutes to less than an hour, sometimes as little as 30 minutes.
**TABLE 2.1**

**CELL YIELD FROM DISSOCIATED CEREBELLMUM**

<table>
<thead>
<tr>
<th>Number of Experiments</th>
<th>Age (days)</th>
<th>Cells (X10^-6/g wet wt.)</th>
<th>DNA Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Original</td>
<td>Modified</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>193 ± 8.8</td>
<td>154 ± 3.8</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>233 ± 29.2</td>
<td>204 ± 27.8</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>263 ± 20.2</td>
<td>337 ± 26.7</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>343 ± 12.9</td>
<td>378 ± 14.8</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>371 ± 63.4</td>
<td>512 ± 69.4</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>377 ± 19.7</td>
<td>490 ± 66.4</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>285 ± 2.2</td>
<td>421 ± 2.5</td>
</tr>
</tbody>
</table>

* See Methods for experimental details

Results expressed as mean ± S.E.M.

\[ p \leq 0.05 \]
In Table 2.1 a comparison of the two methods is made in terms of the total cell yield and DNA recovery obtained at postnatal ages ranging from two to fourteen days. The total cell yield was based on a count of all particles of equivalent spherical diameter >5.5μm. (The Coulter counter measures particle volume and therefore calculation of diameter involves an assumption that the cells are approximately spherical.) Over the age range of 6 to 14 days the total number of cells obtained per gm. wet weight of starting tissue was substantially greater using the modified isolation procedure. DNA recoveries were based on % DNA recovered in the cell suspension compared to whole cerebellum, and varied from between about 30 to 50% using the modified method. Large comparative differences of between 15-25% were seen using animals of 6, 10 and 14 days of age which favoured use of the modified procedure. Only with 2 day rats did the original method produce significantly higher DNA and total cell yields.

However these changes in yield were not equally distributed among different sizes of cells. Five different size classes A - E are defined in Table 2.2; they result from the unit gravity sedimentation work described in the next Chapter and it is sufficient here to state that the A range represents debris, B and C are the size of granule cells, D is the size of inhibitory interneurons, and E represents the large neurons, mainly Purkinje cells. Figure 2.1 shows a comparison between the two methods of the yield in cells per gm. wet weight in each size class at each age.
**TABLE 2.2**

PARTICLE SIZE ANALYSIS OF CEREBELLAR PERIKARYA

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume Range (μm³)</th>
<th>Diameter Range (μm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>&gt;1600</td>
<td>&gt;14.5</td>
</tr>
<tr>
<td>D</td>
<td>500 - 1600</td>
<td>10.0 - 14.5</td>
</tr>
<tr>
<td>C</td>
<td>250 - 500</td>
<td>8.0 - 10.0</td>
</tr>
<tr>
<td>B</td>
<td>130 - 250</td>
<td>6.5 - 8.0</td>
</tr>
<tr>
<td>A</td>
<td>80 - 130</td>
<td>5.5 - 6.5</td>
</tr>
</tbody>
</table>

* Equivalent spherical diameter
Figure 2.1. The total number of cells ($\times 10^6$) per gm. wet weight of whole cerebellar tissue is given for the size ranges A-E over the postnatal period of 2 to 4 days. See TABLE 2.2 for the definition of these designations, and RESULTS section for cells identified in these fractions. The open bars represent results with the original method; closed bars are results obtained using the modified method. Results are expressed as mean values $\pm$ SEM, n = 2-12.
studied. In 2 and 4 day old animals it is clear that there was little difference between the methods in the yield of any of the particular designated cell sizes. However, in perikaryal suspensions obtained from rats of 6, 8 and 10 days of age, the modified method favoured selection of the large C, D and E cell types. Later in development at 12 and 14 days of age, use of the modified method resulted in increased yields of only C and B cell sizes but no longer were there any differences in yields of the larger D and E cells. Note also that at these later ages more debris was produced with the modified method.

DISCUSSION

Cohen's method yields a preparation of cells which are by several criteria more well preserved than those from most bulk isolation procedures (Wilkin et al., 1976) and therefore may more confidently be employed in in vitro biochemical studies. However, for the purposes of the work to be described in subsequent chapters which involves preparation of primary cultures the most important comparison is with other culture-oriented cell dissociation methods.

The important advantages of Cohen's method as a basis for culture are the relative and total yield. The relative yield is high — up to 50% of tissue DNA is recovered in cell perikarya and therefore the composition of the cell suspension must be broadly representative of the proportions of cell types in situ.
In addition, the method works well in cerebella from 8-day old rats, older than the embryonic or newborn animals used in most brain cell culture systems. This has the important practical consequence that the total yield is also high, an average of $20 \times 10^6$ cells from a single animal or $250 \times 10^6$ cells from a normal litter.

Several distinct advantages are apparent from the results using the modified method. Compared to the original the modified procedure has been greatly simplified in that only three solutions are required and are made from the same basic medium of Ca$^{2+}$ free Earle's Basic Salt Solution containing glucose and BSA. Eliminating the use of EDTA, which is potentially cytotoxic, combining the action of DNase with trypsin inhibitor, and reducing the trituration procedure decreased the perikaryal preparation time from about two hours to less than one hour. As demonstrated here there was an increased cell yield, preferentially in the cells of the larger size ranges (>10μm, probably representing the larger inhibitory interneurons and Purkinje cells). Electron microscopic evaluation (by K. Tear) of low and high power fields of the perikarya prepared by both methods showed the same excellent ultrastructural preservation that had previously been demonstrated (Wilkin et al., 1976; Cohen et al., 1978). One final note of evidence from another laboratory suggesting that cell preservation may be superior using the modified procedure comes from the observation that the total cell suspension prepared by this method shows a 50-100 fold increase in cyclic GMP concentration upon addition of glutamate. No glutamate stimulation was observed in cells prepared by the original method (Cohen and
Considering the great reduction in preparation time it is perhaps surprising that the improvements in yield using the modified method are quite modest. This is regarded by this author as tending to vindicate the choices of solutions used and general procedures adopted by Cohen et al. Clearly if a 20% increase in yield results from a 60% reduction in preparation time there is little evidence of progressive damage to the cells due to their presence in suspension in glucose and BSA-supplemented bicarbonate buffers. The crucial practical advantages of the modified method have turned out to be the reduction in time required for preparation of solution and for the cell preparation itself. As this method forms the first step of virtually all of the experiments which provide the data described in this thesis, the saving of time and labour is considerable. The method is highly reproducible, probably due to the use of minimum enzyme concentrations under controlled conditions. Figures for the proportion of successful cell preparations are rarely given in the culture literature (but see Messer, 1977a, who mentions 30%), but for this method would be about 90% of cell preparations which result in acceptable cultures. It is the author's general impression from discussions with other workers that this is an unusually high level of reliability. This is likely to result from the cell dissociation method itself, rather than vagaries of the subsequent culture conditions, since most laboratories report that failures tend to involve a whole cell preparation.
In summary, some essential features for cell preparations from CNS tissue at this stage of development seem thus to be: (i) use of trypsin, but in a carefully controlled manner using a minimum concentration and followed by a trypsin inhibitor (ii) elimination of EDTA (iii) minimum overall time of preparation (iv) all solutions carefully buffered, and supplemented with protein (BSA) and glucose. The modified version of Cohen et al's controlled trypsinisation method offers three advantages:— brevity, high relative yields of cells — giving a representative preparation, and high total yield — giving ample material for biochemical studies.
The brain is clearly a tissue of great heterogeneity, any particular area is composed of several different cell types, and there are great differences in both form and function from one area to another. This structural complexity imposes an important constraint on the interpretation of biochemical measurements made on the whole brain, or even on a more specific region such as cerebral cortex or cerebellum. Such measurements can indicate only the sum of events taking place in many partially interconnecting compartments (see Rose, 1972). There are several levels of compartmentation; including sub-cellular divisions into nucleus, mitochondria and other organelles, and for neurons, into cell body, cell processes and synaptic terminals. However a higher level of compartmentation exists which must be taken into account, and that is between the different cell types, the most fundamental division being between neurons and glial cells.

There are two possible approaches to overcoming the problem for the biochemist of the cellular heterogeneity and complexity of the brain. One is to use techniques, such as quantitative histochemistry, autoradiography, or immunofluorescence which give information on individual cells in the whole tissue. The second is to physically separate the different cell types and study them in isolation. The
dissociation and separation of brain cells, initially simply into fractions of neurons and glial cells, is an attractive approach which appears ideal for analysis of many unresolved questions concerning the interactions of these cell types. Many groups of workers have adopted this approach and achieved fractions of separated neurons and glia. However the ideal way to obtain separated cells from the brain has yet to be found, as is indicated by the variety of different procedures which have appeared, and which remain in use. To compare in detail the different methods for neuron/glia separation is beyond the scope of this introduction and has been adequately covered elsewhere (Johnston and Roots, 1970; Sinha and Rose, 1971, Sellinger et al. 1971; Roots and Johnston, 1972; Poduslo and Norton, 1975; Hamberger and Sellstrom, 1975).

The history of cell separation in the nervous system actually began, according to Roots and Johnston (1972), in 1865 when Deiters published drawings of neurons which he had dissected by hand from the anterior horn of the spinal cord and other parts of the nervous system. Such microdissection techniques, combined with sensitive biochemical methods, have continued to be used by workers such as Lowry (1953) and Hyden (1960, Hyden and Pigon, 1960). A method based on hand selection of neurons from a suspension of cells produced by sieving of the tissue through fine nylon meshes yielded neurons in greater numbers (300 per hour) but still insufficient for routine biochemical investigations (Roots and Johnston, 1964).

The first procedure for the isolation in bulk quantities of fractions enriched in neurons and glia was by Rose (1965) and many different methods have been put forward since that time. The Rose procedure and similar methods (Satake et al. 1968, Blomstrand
and Hamberger, 1969; Flangas and Bowman, 1970; Nagata et al., 1974) involve disrupting the chopped or minced tissue by filtration through fine nylon or steel meshes in a buffered saline medium and then separating the resulting suspension by high-speed centrifugation on discontinuous Ficoll gradients. A second group of methods use filtration through meshes in a phosphate buffer medium with Ficoll or bovine serum albumen and high concentrations of fructose and glucose (Iqbal and Tellez-Nagel, 1972; Jones et al., 1971). This is often preceded by an incubation with trypsin at 37 °C (Norton and Poduslo, 1970; Giorgi, 1971; Hemminki, 1972) and the final separation is by low-speed centrifugations on sucrose gradients.

A somewhat different procedure is that of Sellinger et al. (1971) which involves sieving the tissue in a hypotonic medium containing 10mM Ca++, BSA and polyvinylpyrrolidone (PVP) followed by a series of centrifugations on sucrose and Ficoll gradients. A modification of this procedure was used by James Cohen and his collaborators to isolate cells from the cerebellum of adult mice (Cohen, Mares and Lodin, 1973). Other investigators have modified existing centrifugation methods to produce separated cell fractions from the cerebellum (Yanagihara and Hamberger, 1973; Hazama and Uchimura, 1974; Sellinger et al., 1974).

The structural integrity of neuron/glia preparations, especially that of the external plasma membrane, is inextricably linked to any consideration of their functional attributes. The ultrastructural preservation of cells isolated from brain by methods
of this sort has long been a matter of some difficulty. Roots and Johnston (1964) found many dissimilarities between neurons in situ and those prepared by a sieving technique, including the lack of a plasma membrane. The authors of all the more widely used techniques have stated that preservation of ultra-structure is usually less than perfect, particularly with regard to discontinuities in the plasma membrane (e.g. Rose, 1972, Poduslo and Norton, 1972; Sellinger and Azcurra, 1974; Hamberger and Sellstrom, 1975 and further discussion see Johnston and Roots, 1970). The most detailed ultrastructural examination of bulk isolated neurons, astroglia and oligodendroglia is by Raine et al. (1971) on cells isolated by the methods of Norton and Poduslo. They claimed that the plasma membrane generally remained intact but cellular organelles not infrequently displayed swelling, vesiculation and rarefaction. The high power electron micrographs published by other laboratories (Rose, 1967, Johnston and Sellinger, 1971, Sellinger and Ascurra, 1974, Hamberger et al. 1975) although too few in number to give a definitive result, indicate severe changes in ultrastructure compared with cells fixed in situ.

Electron micrographs of the cerebellar cell suspension prepared by the method of Cohen, Mares and Lodin (1973) indicated a similar severe degree of ultrastructural damage (Wilkin, Balazs, Wilson, Cohen and Dutton, 1976). Small cells were almost completely devoid of cytoplasm and large Purkinje neurons showed extensively swollen mitochondria, the endoplasmic reticulum being vesiculated and greatly distorted and only fragments of plasma membrane remaining. It is
important to note that these cells were of apparently excellent appearance in phase contrast micrographs, the nucleus and nucleolus were clearly outlined and the cytoplasm seemed "richly organised" (cf. Sellinger et al. 1971, Johnson and Sellinger, 1971).

Despite these limitations, bulk preparations of neurons and glia (or neuropil, as some authors now prefer to denote this fraction to take account of the presence of synaptosomes and axonal or dendritic fragments; Rose and Sinha, 1970) have been used with considerable success for biochemical studies of the brain covering many aspects of metabolism. One example, which demonstrates the usefulness of the technique, is the finding that neurons show a 2 to 3 fold higher rate of amino acid incorporation into protein than glia/neuropil when isolated one hour after an \textit{in vivo} injection of labelled amino acid (Blomstrand and Hamberger, 1969, Rose and Sinha, 1974). The ratio declined to below unity after four hours, suggesting a more rapid decay of neuronal perikaryal proteins. This could be due to turnover within the perikaryon but has been argued to be more probably a reflection of transport of protein from the perikaryon to the cell processes (Rose and Sinha, 1974). This interpretation supports the \textit{in vivo} autoradiographic evidence of Droz and Koenig (1970) and suggests that the predominant portion of the rapid protein synthesis known to occur even in adult brain is located in the neuronal perikaryon and followed by rapid transport to the remainder of the cell.

In this example the neuron/neuropil separation was used to analyse the results of an \textit{in vivo} experiment. It is in the \textit{in vitro}
expression of complex functions that we can expect the damage which may have been sustained during isolation to limit the usefulness of these cell preparations. Hamberger and Sellstrom (1975) expressly state that bulk isolated cells are not suitable for tissue culture studies or the maintenance of in vivo membrane potentials. There is no evidence to contradict this. Poduslo and McKhann (1977) have reported the "maintenance" of bulk isolated neurons in vitro for 24 hours without loss of "metabolic activity" (incorporation of precursors into protein and RNA). This may be a useful system, but there is no indication that such cells are capable of attaching to a culture substrate, extending processes and fully recovering their cellular capabilities.

It was as a consequence of the poor ultrastructural preservation found in cells prepared by the method of Cohen, Mares and Lodin (1973) that James Cohen and his collaborators developed the controlled enzymatic tissue dispersion method for the isolation of cells from the cerebellum which is described in the previous chapter and elsewhere (Cohen et al. 1974, Wilkin et al. 1976). This preparation of cells is notable not only for its biochemical integrity by several parameters, but also for the high degree of ultrastructural preservation achieved. The authors show high power electron micrographs of individual cells and in addition low power survey
electron micrographs. These last are essential to gain a true impression of the general level of structural preservation in a cell population. The apparently excellent condition of these cells, including intact plasma membranes and mitochondria, led to the hope that they would form a good basis for cell culture if a method of cell separation could be found which did not cause further damage.

**Unit gravity sedimentation**

All the methods for bulk isolation of separated cell fractions which have been mentioned depend on centrifugations on density gradients, that is the cells are separated on the basis of density. A different approach has been described for non-neural tissues by Peterson and Evans (1967) and in greater detail by Miller and Phillips (1969). This is a method based on velocity sedimentation at unit gravity (1g) and leads to a separation by cell size rather than cell density.

The technique was first exploited in the nervous system by James Cohen (Cohen, Mares, Lodin, 1973) who used unit gravity sedimentation to separate cells prepared from adult mouse cerebellum by a modification of the method of Sellinger et al. (1971). The differences in size in the cerebellum between different cell types were particularly great and Cohen et al. were able to achieve good enrichments of the small proportion (1%) of very large Purkinje neurons from the unseparated cell suspension. The resulting preparation was well-suited to answer the biochemical question they were asking of it. They were able to show that Purkinje cells
contained the normal diploid quantities of DNA per cell. This direct biochemical data weighed heavily against the common suggestion, based on cytophotometric measurements using the Feulgen reaction, that Purkinje cells were polyploid (e.g. Lentz and Lapham, 1970). However as detailed above, Cohen and others have subsequently shown that the cells prepared by this method are heavily damaged at the EM level and are thus unsuitable for determination of more labile biochemical properties and certainly not viable in culture (Wilkin et al. 1976).

In the same year, the method of unit gravity sedimentation was applied to trypsinised cells of newborn mouse cerebellum by Barkley et al. (1973). These workers were using a separation chamber which permitted only $5 \times 10^6$ cells to be processed, giving very low yields in the less numerous fractions. However they clearly showed the possibility of separating proliferating cells at different stages of the cell cycle, and also of enriching Purkinje or other large early-forming neurons. They gave no data on the condition or viability of the cell fractions other than the observation that cells from some fractions will form aggregates after 24 hours in vitro. This group seems not to have pursued this work after a promising beginning. Velocity sedimentation at $1g$ has also more recently been used to enrich large motoneurons in spinal cord cell suspensions before culture (Berg and Fischbach, 1978).

Unit gravity sedimentation is a particularly gentle separation procedure which may permit the retention of viability when applied to the total cell suspension from cerebellum described in the previous chapter, which contains cells of widely different sizes. The results
of the application of the technique to this cell suspension form
the main part of the present chapter.

In passing it must be mentioned that a completely different
approach to the separation of cells from the nervous system is
represented by the small number of groups who have achieved cultures
enriched in particular cell types by manipulation of the culture
conditions. This work is reviewed in the next chapter.

**Principle of velocity sedimentation at unit gravity**

The general arrangement of layers in a sedimentation chamber
immediately after loading is shown in Figure 3.1a. The cells are
allowed to sediment from the narrow band in which they are initially
loaded, down through a gradient of BSA. The sedimentation velocity
\( S \) of a spherical cell falling through a fluid under the influence
of gravity is given by:

\[
S = \frac{2(\rho - \rho')}{9\eta}gr^2
\]

where \( \eta \) is the coefficient of viscosity of the fluid medium, \( \rho \)
and \( \rho' \) the densities of the cell and fluid medium respectively,
g the acceleration due to gravity, and \( r \) the radius of the cell.

Under conditions where the difference between \( \rho' \), the density
of the medium and \( \rho \), the density of cells is always much greater
than any variation in cell density, the velocity of sedimentation of
any cell is thus proportional to its \((\text{radius})^2\). The separation is
therefore on the basis of cell size; it can also be shown to be largely independent of any plausible variation in cell shape (Miller and Phillips, 1969). During a two hour run under the conditions described below, a cell of diameter 9μm will sediment approximately 8mm while a cell of diameter 16μm will sediment about 32mm.

It must be emphasised that the purpose of the gradient of BSA is purely to prevent convection and mixing of adjacent layers, particularly during loading and unloading the chamber. The density of the medium varies from 1.004 to 1.009 gm/cm³. Nucleated cells typically have a density of about 1.06 gm/cm³ (Miller and Phillips, 1969) and so the gradient does not significantly affect the sedimentation velocity of most cells. The contrast with density gradient centrifugation is here apparent. In that case continuous or discontinuous gradients are set up so that, on prolonged centrifugation, cells of differing densities will reach the position at which they "float", i.e. at which ρ = ρ' in the equation above. However, if the unit gravity chamber were allowed to sediment to equilibrium all the cells would fall to the bottom for they are all more dense than the fluid medium. A centrifugation procedure to separate such large particles as cells by velocity sedimentation would have to be for very short times or at very low g forces. Neither seems practicable. One other possibility is revealed by a glance at the above equation. If the viscosity of the medium could be increased, without increasing the density, then this would partially counteract the massive increase in g given by even a low-speed centrifugation and might allow size-based velocity separations in
centrifuge runs of reasonable length. Compounds such as sodium metrizoate exist which will provide such an increase in viscosity but their use might prejudice cell viability and might not overcome the problem of "streaming" (see below). For the present, the method in use seems adequate despite the rather long time of the procedure and the fixed limit on cell numbers.

The practical limit on the number of cells that can be loaded is dictated by the phenomenon of "streaming". If the cell band is at too high a concentration, then it takes on the appearance of an "upside-down grass lawn" (Miller and Phillips, 1969) with individual streamers of cells extending 1 cm or more downwards into the gradient. This phenomenon is not well understood; it resembles a convection effect and yet the gradient satisfied all the conditions for convective stability. Cell clumping is not a likely explanation as cells remain as single entities at concentrations well above the streaming limit. What is clear is that if streaming occurs the separation is completely disturbed and therefore cells must always be loaded at concentrations below the streaming limit. However if the cell band is too wide then clearly a sharp separation cannot be expected as the cells begin their passage down the gradient from different starting points. This therefore limits the total number of cells which can be separated.

METHODS

Apparatus

The unit gravity sedimentation chamber which was used for this
work is shown in Figure 3.2. It was manufactured by the workshops at MRC Laboratories, Carshalton to our specifications. The design is basically similar to that used by earlier workers and was further developed by James Cohen. It was machined from a single block of perspex to the dimensions shown and carefully polished. The essential features of the chamber design include the cone angle of 30° to the horizontal, the small stainless steel baffle over the entry port to prevent mixing, a device for levelling the chamber, and the high degree of polish of the chamber which minimises retardation of cells by surface irregularities on loading and unloading.

Ancillary apparatus included:

(i) a 50 ml syringe barrel for loading the initial buffer layer and the cell layer.

(ii) An LKB gradient maker for generating the gradient of 0.5 - 2.0% BSA.

(iii) An LKB Ultrorac fraction collector.

(iv) Technicon tubing for all connections, which was renewed at regular intervals.

Procedure

For non-sterile preparations, several steps of this procedure can be omitted including sterilisation of apparatus, filtration of
Buffer layer - 50ml. CMF-KRB

Cell layer 10-50ml 0.2% BSA in CMF-KRB containing 40μg/ml DNase

600ml gradient of BSA in CMF-KRB

Figure 3.1a: Appearance of chamber after loading.

Double layer of turbidity

Debris

Cells

Figure 3.1b: Appearance of chamber after 2 hours separation.
vent plugged with cotton wool for sterile work

50ml syringe barrel for addition of buffer layer and cell band

lid

chamber

spirit level

Technicon tubing connections

Tripod legs with levelling adjustment

Figure 3.2a: Diagram of chamber and ancillary apparatus

Figure 3.2b: Photograph of chamber and ancillary apparatus
D Li

solutions at 0.22μm maximum pore size and capping of tubes.

All apparatus was sterilised with 70% alcohol and allowed to dry in a laminar flow hood, except for the chamber itself and the plastic collection tubes which were sterilised by exposure to UV light for 1 hour. All solutions were pre-filtered by vacuum through 0.45μm Millipore filters, this is necessary to reduce background counting in the Coulter counter. The gradient solutions (300 ml each of 0.5 and 2.0% BSA in CMF-KRB) were filtered by vacuum at 0.22μm, transferred to the sterilised gradient maker, covered and set up in cold room. The 0.2% BSA cell suspension solution was dispensed by a 50 ml syringe through a sterilised 3 cm Swinnex filter holder containing a 0.22μm Millipore filter. 50 ml of Ca^{++}, Mg^{++} free Krebs Ringer buffer (CMF-KRB) was also filtered at 0.22μm by use of a syringe and Swinnex. The composition of the fluid medium is important for maintenance of cell viability. The presence of glucose and a protein, either serum or BSA seems important. Gradients based on Ficoll were found to give much inferior cell recoveries. Miller and Phillips (1969) report loss of viability with gradients based on sucrose. It is also necessary to run separations at 4 °C for optimum viability.

**Loading**

1. 400μg of DNase (Sigma) was dissolved in 10 ml of 0.2% BSA solution. The cell pellets obtained after the final spin of the cell isolation procedure (described in the previous chapter)
were resuspended in this DNase solution by gentle pipetting, pooled and counted (Coulter counter, see below). A proportion of this total cell suspension was then diluted with 0.2% BSA to give the final cell numbers and concentration required. The streaming limit for cerebellar cells under the conditions shown is about $4 \times 10^6$ cells/ml. 50 ml of medium results in a band about 2.5 mm wide in the chamber shown, this is the upper limit for reasonably clean separations. Thus the maximum number of cells which can be processed in one run is slightly under 200 million. The maximum loading normally used was 185 million cells in 50 mls giving a cell concentration of 3.7 million/ml.

2. The chamber was loaded in the cold room at a rate of about 20 ml/min; Figure 3.1a shows the arrangement of layers. The 50 ml stabilising layer of buffer (CMF-KRB) was loaded first by use of the 50 ml syringe barrel. It was important as the fluid first entered the chamber to ensure no bubbles were trapped under the baffle which might later escape and disturb the layering of cells.

3. The cell layer was allowed to flow in immediately after.

4. As the last of the cell layer left the 50 ml syringe barrel, the tube was constricted, disconnected and reconnected to the gradient maker. It was important during this operation to prevent bubbles entering the tube.
5. The gradient was allowed to run in and the runs were timed from the moment the cell layer left the cone.

**Collection**

After the run time (usually 2 hours) the chamber was emptied at about 15 ml/min and fractions were collected in 10 ml sterile plastic conical tubes (Falcon or Sterilin). As each rack of 10 tubes was collected, the caps were replaced and the racks transferred to a fridge near the Coulter counter.

**Cell counting and fixing**

To maintain sterility it was necessary to make a transfer in the laminar flow hood of an aliquot from each 10 ml fraction (or a representative selection of them) into another tube. The aliquot was diluted to 10 ml with filtered CMF-KRB, transferred to a 25 ml Coulter counting vial and counted on a Coulter counter model ZB using a 140\(\mu\)m orifice tube. Aliquots were generally 1 ml or 0.2 ml giving dilutions for counting of 1 in 10 or 1 in 50 depending on the cell numbers expected in the fraction. Several counts were made in different size ranges by resetting the lower and upper threshold on the model ZB. Size distribution plots were produced using a Coulter Model P64 Size Distribution Analyser coupled with an X-Y plotter.
Further processing of fractions

After counting cells were collected by low speed centrifugation (MSE Mistral 4L, 1000 rpm, 180µg, 7.5 min.) The resulting cell pellets were resuspended and pooled as required by gentle pipetting in small volumes of a medium appropriate to the intended use of the cells, for instance culture medium.

Cell suspensions were fixed for light and electron microscopy by adding an equal volume of Karnovsky fixative containing 1% formaldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4. After 30 minutes at room temperature, the fixed cells were transferred to polyethylene conical tubes and packed by centrifugation for 15 seconds at 10,000g in a Beckman Microfuge 152. The tips of the tubes were sliced off, halved in the plane of sedimentation and post-fixed in 1% osmium tetroxide in veronal acetate buffer for 1 hour at 4 °C. The pelleted material was embedded in Durcupan resin and sectioned for light microscopy (~1µm sections) or electron microscopy. Processing for EM was by G. Wilkin, and the microscopy was performed and analysed by G. Wilkin and F. Hajos at the MRC Developmental Neurobiology Unit, Carshalton.

In vitro incorporation of labelled precursors into separated cell fractions was determined by incubating cells resuspended in KRB with 10 Ci/ml 6-^3^H-Thymidine (2µCi/mmole) or 2.5µCi/ml 3^H-glutamate (250 µCi/mmole) at 37 °C under a 95% O\textsubscript{2}/5% CO\textsubscript{2} atmosphere in siliconised scintillation vials in a shaking water
bath. At various time points, duplicate samples were removed and added to 1 ml of ice-cold 10% trichloracetic acid in the presence of 300μg of BSA as carrier. The precipitate was washed three times with 10% TCA, dissolved in 0.5 ml Nuclear Chicago solubuliser, and counted after addition of 10 ml of 4:1 toluene/methoxyethanol containing 6 g/L 2,5-diphenyloxazole.

**In vitro** incorporation of $^3$H-Thymidine before separation was accomplished by incubating the total cell suspension at $20 \times 10^6$ cells/ml in chamber loading medium (0.2% BSA in CMF-KRB with 40μg/ml DNase) in the presence of 25μCi/ml of 6-$^3$H-Thymidine (2 Ci/mmol) at 37°C for 15 minutes prior to loading the chamber in the normal manner. Fractions were processed for liquid scintillation counting as above.

**In vivo** incorporation of $^3$H-thymidine was by intra-peritoneal injection of 80μCi of 6-$^3$H-thymidine (5 Ci/mmol) per 10g, 6-day old rat at 90 minutes before killing. Cells to be examined by autoradiography were smeared on acid-washed gelatine-subbed slides. Smears were heat-fixed and coated with Ilford K2 emulsion, exposed in the dark at 4°C for 2-4 weeks, developed for 8 minutes at 22°C with Kodak D19 developer, and fixed for 10 minutes with Ilford Hypam. Slides were stained with haematoxylin and eosin, estimates of percentage labelled cells were based on counts of 1000 cells per fraction. This work was performed by K. Tear.
CALCULATION OF COULTER RESULTS

A major part of the results section is devoted to a size analysis of the fractions obtained from the chamber sedimentation, demonstrating how this analysis led to the definition of particular size classes known as A-E, which could subsequently be characterised according to morphological and other criteria. As a preliminary to this section, the principles of the Coulter counter, and of the calculation of results from it, will be briefly set out here.

The Coulter counter

In the Coulter counter, a suspension of particles in an electrolyte (CMF-KRB) is sucked through a small round orifice of diameter, in this case, 140 μm. The resistance across the orifice is measured and the momentary change in resistance as an individual particle passes through is detected. The size of the electronic pulse generated in this way is directly proportional to the volume of the particle. The machine counts the number of pulses occurring while a fixed volume (in this case 0.5 ml) of suspension is sucked through the orifice. The size range of pulse which the machine will count is dictated by the setting of a lower and upper threshold. The threshold scale \( T \) is calibrated in arbitrary units of 0-100 which are proportional to volume on any given setting of the other relevant controls. These are in particular, the aperture current \( I \) and the amplification factor \( A \) (the upper threshold can also be set at \( T = \infty \) i.e. removed).
These quantities are related by the expression:

\[ V = K \cdot A \cdot I \cdot T \]  
(The Coulter Equation)

where \( V \) is the volume of a particle (in \( \mu m^3 \)), \( T \) is the height on the threshold scale of the pulse generated by that particle, \( A \) and \( I \) are as above, and \( K \) is the calibration constant which converts to \( \mu m^3 \) from the arbitrary units in which \( A \), \( I \) and \( T \) are expressed. \( K \) is determined by calibrating the machine with latex beads of a known diameter and remains unchanged unless the orifice tube is replaced.

Thus the counts obtained with specific settings of the lower and upper threshold can be converted to counts per ml of suspension in a known range of volume in \( \mu m^3 \) (or range of diameter in \( \mu m \) if the particles can be assumed to be spherical).

**Size distribution plots**

To aid in analysis of the Coulter results, the series of electronic pulses generated by the counter were further processed by a Coulter Size Distribution Analyser Model P64, coupled with an X-Y plotter. A typical set of curves generated by this equipment is shown in Figure 3.3. The abscissa is \( T \), the threshold, and the ordinate is an arbitrary scale which, for any single curve, is proportional to the number of cells at a particular \( T \) value (i.e. volume).

Each curve is essentially a histogram. The P64 divides the threshold range into 64 equal "bins" and accumulates counts in each until one reaches the level which has been designated full scale.
Figure 3.3: Reproduction of original Coulter P64 data which is re-calculated in Figure 3.4.
It then ceases accumulation and plots the resulting curve. In the case of a cell population which contains cells of widely differing sizes such as the one which has been used here as an example, it is necessary to plot more than one curve to cover the range. For instance, in Figure 3.3 the solid curve was plotted with A set at 1 and I set at 1/8, so the threshold range $T = 0 - 100$ represents a volume range $V = 0 - 820 \, \mu m^3$. The broken curve was plotted with A set at 2 and I set at 1/4 so the threshold range $T = 0-100$ represents a volume range $V = 0 - 3280 \, \mu m^3 (K = 65.6 \, \text{for this orifice tube})$. In each case a level ("P") was set on the P64 below which all counts are ignored. This prevents the large number of counts in small size "bins" due to sub-cellular debris swamping the machine and preventing the accumulation of counts in larger size ranges.

In the example shown in Figure 3.3 the level of statistical variation ("noise") is quite high. Most curves are generated from fractions which have a higher cell concentration than this example; when more cells are available for counting, then smooth curves without significant noise can be generated.

**Generation of a volume curve**

By taking into account the area under each curve and the number of cells (per ml of suspension) present in the volume range represented by each curve, it is possible to convert the height of a particular point on the curve to a percentage of the total cells per ml which is represented by that height. Thus the two curves become
expressed on a common basis and can be reconciled to form a single plot of % cells against volume. As the curve represents a histogram any such "% cell" figure is actually based on the number of cells in a notional volume channel ("bin") of a particular width which centres on the point in question. A volume channel width of 32.8 µm³ was selected for all the calculations as this represented a whole number of units on the arbitrary T scale. However, the width of channel selected only affects the numerical values on the ordinate, and does not affect the shape of the curve.

Figure 3.4a shows the curve of % cells (Cᵥ) against volume generated when the curves shown in Figure 3.3 are reconciled in this way (variations due to noise have been ignored).

The calculation required is:

\[ Cᵥ = \% \text{ of total cells in a volume channel centred on threshold value } T. \]

\[ = \frac{\text{height of curve at } T \times \text{ standard channel width} \times \text{cells/ml represented by curve}}{\text{area of curve}} \times \frac{\text{total cells/ml}}{\text{cells/ml}} \]

As they cancel out, the units of height, width and area may be arbitrary, in practice the units of the Coulter plots.

**Generation of a diameter curve**

The curve (histogram) of cell number against volume is not the most satisfactory way to display the cell size distribution as it
% of total cells in volume channel of width 32.8 µm³

Figure 3.4a: Volume-based cell size distribution generated from data shown in Figure 3.3

% of total cells in diameter channel of width 0.2 µm

Figure 3.4b: Diameter-based cell size distribution generated from data shown in Figure 3.3
compresses the small cells into a few volume channels at the low end of the range and spreads larger cells over many at the high end. Conversion to a curve based on diameter gives a more balanced picture which is intuitively easier to understand although, of course, no more intrinsically valid.

Conversion of the values of the abscissa from volume to diameter is straightforward and involves the assumption that the particles are spherical, which is approximately true in the case of cells in this system. However the conversion of the ordinate values, of % of the total cells which are of a particular size, is not so self-evident. This is because the diameter channels, which make up the histogram of % cells against diameter, are related in a non-linear fashion to the volume channels, which make up the histogram of % cells against volume; the conversion from one to the other involves a factor of \((\text{diameter})^2\). The details of the calculation are shown below.

A comparison of Figure 3.4b with Figure 3.4a shows how the curve against diameter gives a better impression of the relative importance of the two cell populations in the example chosen. Although a careful comparison of the total cell numbers in each peak, by measuring the areas under the peaks would give the same answer whichever plot was used.

Calculation:–

For conversion of per cent of total cells, \(C_v\), in a volume channel of width 32.8 \(\mu m^3\), to the percent of total cells, \(C_d\), in a
diameter channel of width 0.2 μm when the diameter is \( d \) microns:

\[
C_d = C_v \times \frac{0.2}{32.8} \times \frac{\pi d^2}{2}
\]

RESULTS

The appearance of the chamber after loading is illustrated in Figure 3.1a. Figure 3.1b shows the appearance after two hours sedimentation. A double band of turbidity can be seen at this stage. The upper band is the material which has not sedimented significantly and consists of small particles of sub-cellular debris. The lower band is the region containing the greatest numbers of cells and consists of small cells. The larger cells have sedimented much further but are at too low a concentration to be visible to the naked eye.

The appearance of the column is reflected in the numbers of cells found in each fraction by Coulter counting (Figure 3.5). Particles are counted as 'cells' if they are greater than 5.5 μm in diameter. In Figure 3.5 the fractions are shown in order of collection, therefore the direction of sedimentation is from right to left. The peak of cell numbers can be clearly seen and a smaller second peak, corresponding to the upper turbid band of sub-cellular debris, is also seen. The number of particles in the debris peak will be very high, however this analysis only shows the largest of those particles (>5.5 μm) which fall into the 'cell' size range.
Figure 3.5: Total number of 'cells' (particles greater than 5.5μm in diameter) found in each 10ml. fraction collected from sedimentation chamber. Direction of sedimentation is from right to left.
Cell size distribution of unseparated cells

An original cell size distribution plot of the total cell suspension (TCS) is shown as Figure 3.6. The most common cell type can be seen to be small with a volume of 193 µm$^3$ (diameter 7.2 µm). These are mainly the small granule cells seen as the most common population in the TCS by microscopy (see previous chapter). It is encouraging that the cell population is clearly distinct from the population of small debris which can be seen at the left hand edge of this plot. This debris is excluded from other plots shown (e.g. Figure 3.10) by setting the level (P) of the P64 Size Distribution Analyser below which all counts are ignored at T = 10 (equivalent to diameter 5.5 µm).

Cell size distributions of separated cell fractions

Much of the following description of the separated fractions of cells given by the unit gravity sedimentation will involve the use of a series of size-based categories designated A, B, C, D, E and Bp. The definition of these categories was not arbitrary but was based on the results of the first chamber separation to be analysed by the Coulter counter. Subsequent evidence has amply confirmed the selection of these particular categories. In order to make this point clear, some of the results of this first run are shown in Figures 3.7 and 3.8.

20 ml fractions were collected from this chamber run and
Figure 3.6: Coulter P64 plot of TCS. $A = 1, I = \frac{1}{2}$, therefore
abscissa $T = 0 - 100$ represents volume range $V = 0-820\mu m$. $P = \beta$. 
Figure 3.7: Reproductions of original Coulter plots of fractions 8-12 and 17 from the first chamber separation to be Coulter analysed, showing evidence for two discrete populations of cells in this size range. \( A = 1, I = \frac{1}{2} \), therefore \( T = 0-100 \) represents \( V = 0-820\mu m \). Full description in text.
Figure 3.8: Reproductions of original Coulter plots of fractions 1, 3, 5 from the first chamber separation to be Coulter analysed. 

\[ A = 2, \quad I = \frac{1}{4}, \quad \text{therefore} \quad T = 0-100 \text{ represents } V = 0-3280 \mu m \]. Full description in text.
reproductions of the original Coulter P64 plots of five adjacent fractions (numbers 8 - 12) are shown in Figure 3.7. A uniform transition in cell size from one fraction to the next might be expected if there is continuous variation of cell size in the total cell suspension. However there is a clear indication in these plots of two discrete cell species, the larger of which is strongly represented in fraction 8 with a peak of cell numbers at a T value of 42 (volume 345 μm³, diameter 8.7 μm). The smaller species is most strongly concentrated in fraction 12 with a peak of cell numbers at a T value of 23.5 (volume 193 μm³, diameter 7.2 μm). Fraction 12 also contains the highest number of cells of any fraction. Later fractions which are closer to the starting point of the sedimentation have a profile indicating mainly debris present, and an example, Fraction 17 is also shown in Figure 3.7.

From these plots, three size ranges were defined, A was from T = 10 to T = 16, B was from T = 16 to T = 31 with the intention of including the smaller of the two discrete cell species seen, C was from T = 31 to T = 61 in order to include the larger cell species seen most clearly in Fraction 8.

Turning now to the early fractions which contain large cells, Figure 3.8 shows Coulter P64 size distribution plots of Fractions 1, 3 and 5. It is important to note that these plots are over a wider size range than those shown in Figure 3.7; the larger range is obtained by changing the settings of A and T. The top of the C size range occurs at T = 61 on the smaller range plots, and this is
equivalent to a value \( T = 15 \) on the larger range plots shown in Figure 3.8. Two further cell classes were defined, these are D, from \( T = 15 \) to \( T = 50 \) and E from \( T = 50 \) to \( T = \infty \). A setting of \( T = 50 \) in this range is equivalent to a diameter of 14.5 \( \mu m \), and so only the larger neurons (mainly Purkinje cells) would be expected to fall in size class E. An examination of the plots of Fractions 1, 3 and 5 shows no evidence of discrete cell species in this region of larger cells and therefore the division between D and E is more arbitrary than that between B and C. Size classes A - E are specified in detail in Figure 3.9.

**Numbers of cells in size classes**

Once cell classes A - E had been defined, it was possible in each fraction of subsequent sedimentations to make counts of the cells which fell into each of the size classes. This was done by counting between different settings of the upper and lower threshold controls on the main ZB machine. Figure 3.9 shows the data which can be obtained, by this procedure. Each size class is enriched during the separation, to about 50% in some fractions. This represents a 2-3 fold enrichment of B or C, and a 50-fold enrichment of E. In routine use it is no longer necessary to plot size distributions as the peaks of enrichment of particular size classes can be obtained by counts of this type.

Another important feature emerges from Figure 3.9. C size cells show a second peak of enrichment in fractions from just above
Figure 3.9: The relative frequencies of the size classes defined above in each fraction collected from the chamber, showing the peaks of enrichment of each size class. Thus Fraction 20 contains 56% D, 19% C, and 17% E. 6 day rat cerebellar cells.
the B peak in the chamber. This is seen in almost every chamber separation performed. It implies that a population of cells in the C size range are sedimenting at an anomalous position above the B peak, this 'paradoxical' behaviour led to these cells being named Bp. The anomalous sedimentation of these cells implies that they are significantly different in density from the majority of cells. 'Bp' is in some ways an unsatisfactory name, however an alternative name (C') given to it in a publication of this work (Cohen et al. 1978) is too easily confused with C itself, therefore the term Bp will continue to be used here.

Cell size distributions of the peak fractions

As a first step in the characterisation of cell classes A-E, it is possible to look in detail at cell size distributions of the chamber fractions which represent the peaks of enrichment of E, D, C, B, Bp and A (in order of collection from the chamber). Figure 3.10 shows the raw size distribution plots of these peak fractions from a chamber separation of 6 day old rat cerebellar cells. Figure 3.11 shows the reconciled volume-based size distributions and Figure 3.12 shows the diameter-based size distributions. Simple counts of the numbers of cells in each size range are sufficient to identify these fractions for routine work, however it is useful to look at the more clear and detailed picture given by, in particular, the diameter-based curves shown in Figure 3.12.

The fraction enriched in A size 'cells' shows no evidence of a distinct species of cell in this size range, but rather indicates the
Figure 3.10: Reproduction of original Coulter P64 size distribution analyser plots of TCS and peak fractions of a separation of 6-day rat cerebellar cells.

Range 0-820μm³

Range 0-3280μm³

*A second example of an E peak – from a separation of 7 day old rat cerebellar cells.
Figure 3.11: Volume-based size distributions of TCS and peak fractions of a separation of 6-day rat cerebellar cells. The horizontal bars at the top show the position of size ranges A-E. \( C_V \): \% of total cells in a volume channel of width 32.8 \( \mu m^3 \). \( V \): Volume of cells in \( \mu m^3 \). \( E^* \): A second example of an \( E \) peak from a separation of 7 day rat cerebellar cells.
Figure 3.12: Diameter-based size distributions of TCS and peak fractions from a separation of 6-day rat cerebellar cells. The horizontal bars at the top show the position of size ranges A-E. $C_d$: % of total cells in diameter channel of width 0.2 $\mu$m. $d$: cell diameter in $\mu$m. E*: A second example of an E peak from a separation of 7-day rat cerebellar cells.
likelihood that this fraction consists of debris, the largest of which fall into size range A (5.5 - 6.5 μm). By contrast, the peak fractions of B and C each contain a well defined population of cells with the highest number of cells being of μm diameter in B, and μm diameter in C. The diameter-based curve of fraction Bp (Figure 3.12) particularly clearly shows the 'paradoxical' presence of a population of larger cells when compared with the faster-sedimenting fraction B.

The curves of D and E, the peak fractions enriched in larger cells show the presence of a significant population of smaller cells in addition to the clear peaks of large cells, this is reflected in the cell counts, and in the microscopic studies of these fractions. However comparison with the cell size distribution of the unseparated cells (TCS) shows the great degree of enrichment of large cells which is seen in these fractions (50% vs. 1%). In addition it should be noted that a 50% level of E cells by number represents more than 80% of the tissue mass present in the fraction due to the several-fold greater volume of E cells. Finally, the E fraction of the example chosen represents the lower end of the range of variation in enrichment of E cells found in chamber sedimentations. Another example, from a different sedimentation run is included in Figures 3.10 - 3.12 to illustrate the higher level of enrichment which can be achieved.

Light microscopy of the peak fractions

Figure 3.13 shows phase contrast micrographs of the total cell
suspension and each of the fractions enriched in a particular size class. Figure 3.14 shows stained semithin (1 μm) sections prepared by fixation and embedding as for electron microscopy. The phase contrast pictures demonstrate marked differences in size and appearance of the cells between different fractions, these differences are emphasised by the greater detail of the stained sections.

Fraction E exhibited an outstanding enrichment of large cells, many of which showed features typical of Purkinje cells such as the eccentric localisation of the pale nucleus and the large nucleolus. Fraction D contained a mixture of large and intermediate size cells with no great enrichment of one particular cell type. Fraction C contained intermediate size cells, a high proportion of which were darkly stained, and including an elevated number of mitotic cells in comparison with the TCS. Very darkly staining small cells were predominant in fraction B. Fraction Bp contained many cells of about the same size as C but which were lightly stained. Fraction A confirmed the prediction of the Coulter results, it consisted of debris, free nuclei, and a few heavily damaged cells.

A comparison of the separated fractions with the TCS indicates the enrichments achieved in some cell types and also shows that the appearance of the different types of cells was not changed during the separation. The proportion of cells excluding trypan blue remained at about the same level, always over 80%, in the separated fractions as in the TCS. An important reservation is resolved by these pictures, they confirm that the large particles seen by the Coulter
Figure 3.13: 9-day rat TCS and separated fractions as labelled. Scale bar 50µm.
Figure 3.14: 6-day rat TCS and fractions as labelled. Note that fraction C contains several cells in mitosis. Scale bar 50μm
analysis to be enriched in Fractions D and E are in fact large single cells, and not aggregates of small cells which would be indistinguishable by Coulter counting.

Electron Microscopy of the peak fractions

Electron microscopy allows a more rigorous assessment of the integrity of the cells, and provides evidence on the identity of cells in the separated fractions. Firstly, there is no ultrastructural evidence of damage during the separation procedure. The cells of the separated fractions exhibit the same high degree of preservation as the total cell suspension (see previous chapter). Electron micrographs of fractions E, Bp and B are shown in Figures 3.15 - 3.17 (EM studies were performed by G. Wilkin and F. Hajos, figures from Cohen et al., 1978). By comparing the ultrastructural appearance of the dominant cell types in the separated fractions with that of cells in cerebellar sections, it was evident during the electron microscopy study that many pointers for cell identification could still be recognised after separation. The proportions of cells of each morphological type were estimated. For comparison, the composition of the total cell suspension was found to be as follows: Purkinje cells - 1%, external astrocytes - 18%, granule cells and differentiating granule cells - 44%, other types (including differentiated interneurons and unidentified cells) - 38% and mitotic cells - 0.7%.

The most frequent cell type in peak E (Figure 3.15) was found to be Purkinje cells (38%). They were well preserved, exhibiting undisturbed
Figure 3.15: Electron micrograph of 6-day rat Fraction E. Note the morphology of the large neurons, see text.
Figure 3.16: Electron micrograph of 6-day rat Fraction B. Description in text.
Figure 3.17: Electron micrograph of 6-day rat Fraction Bp. Description in text.
plasma membranes and many cytoplasmic organelles, including mitochondria which are particularly sensitive to tissue disruption. The nucleus was characteristic of Purkinje cells. It tended to be asymmetrically located at one (axonal) pole of the perikaryon, there were deep nuclear invaginations in the opposite (dendritic) direction, the chromatin was evenly distributed and there was a large centrally located nucleolus. The mitochondria were very numerous and located almost exclusively in the presumed dendritic pole of the cell.

Cells in peak fractions C and B (Figure 3.16) had a similar ultrastructural appearance, and differed only in their size and in the greater frequency of mitotic cells which was observed in peak C, 3% versus 0.7% in the TCS and virtually zero in peak B. The most prominent feature in the cells of peaks B and C was the polymorphous nucleus containing randomly distributed large blocks of condensed chromatin. The nucleus was surrounded by a thin rim of cytoplasm containing a few mitochondria, Golgi apparatus and a rich concentration of free ribosomes. Thus the great majority (80%) of the cells in B and C exhibited features which are typical of external granule neuroblasts or immature granule neurons, although the nuclear invaginations were more pronounced than usually encountered in sections (Palay and Chan-Palay, 1974).

The predominant cells in peak Bp (Figure 3.17) were of the same size as C cells but of quite different appearance. They had light cytoplasm and a large pale oval nucleus with an eccentrically located nucleolus. The chromatin was fairly evenly distributed but often formed a thin
electron-dense apposition along the inner surface of the nuclear envelope. These features are characteristic of cerebellar astrocytes (Palay and Chan-Palay, 1974). A greater proportion of these cells showed signs of damage when compared with other fractions, although the same high proportion (80%) excluded trypan blue.

Electron microscopy of peak A confirmed the light microscopic observations that this is a fraction of damaged cells and subcellular debris.

In vivo incorporation of labelled thymidine

In order to locate the main population of proliferating cells, the external granule cells, 6-day old rats were injected with tritiated thymidine. Ninety minutes later they were killed, and cells were prepared and separated according to the normal procedure. The cell fractions were Coulter analysed and then processed for liquid scintillation counting. Figure 3.18 shows the radioactive counts found in the separated fractions. These data distinguish very clearly between the structurally similar B and C peak fractions. The C peak is seen to be greatly enriched in cells which were in S-phase at the time of cell isolation. This correlates with the microscopic evidence that the C peak is enriched in mitotic cells.

Similar results were obtained from separations in which the cells were labelled with $^3$H-Thymidine for 15 minutes in vitro prior to
Figure 3.18: A sedimentation profile of cerebellar cells from 6 day old rats separated after in vivo incorporation of [$^3$H]thymidine (80 μCi/animal; 5 Ci/m mole) for 90 minutes before killing. The horizontal bars show the peaks of enrichment of the cell size classes.
separation (not shown). Aliquots of the total cell suspension, and of the separated cell peak fractions which had been labelled in this manner were taken and processed for autoradiography. 32% of the cells in the C peak were labelled, compared with 11% in the total cell suspension and 7% in the B peak. 20% of the cells in the D peak were labelled indicating a degree of size heterogeneity in the S-phase cells. The autoradiography clarified the unexpected labelling seen in peak E: in no case were grains seen over the large neurons, but only over the contaminating small cells of similar appearance to those of fraction C.

**Metabolic competence of the separated cells**

The *in vitro* incorporation of $^3$H-Thymidine described above indicates that the cells which are isolated in S-phase maintain their DNA synthetic capacity after isolation, but before separation. The ability of the cells to synthesise DNA after separation was examined by *in vitro* incubation of the peak fractions with $^3$H-Thymidine. As shown in Figure 3.19, incorporation of labelled thymidine proceeded at a linear rate for two hours in the separated fractions as well as in the total cell suspension. The enrichment of fraction C in S-phase cells was again emphasised by these data.

The cells also remain competent in protein synthesis after separation. The incorporation of $^3$H-glutamate into acid insoluble material during incubations of the peak fractions and the TCS is shown in Figure 3.20. Similar results were obtained with different
Figure 3.19: Time course of in vitro incorporation of $[^3\text{H}]$thymidine in the total cell suspension and separated fractions. The incubation medium contained 10μCi/ml $[^3\text{H}]$thymidine (2μCi/mmole).
Figure 3.20: Time course of in vitro incorporation of $^3$H glutamate into protein. The incubation medium contained 2.5μCi/ml $[^3H]$glutamate (250μCi/mmole).
amino acid precursors. The incorporation was sensitive to 50 μg/ml cycloheximide. The differences between fractions are mainly a reflection of different tissue mass per cell. The exceptions to this rule were fraction Bp and fraction A which consistently showed a very low rate of incorporation into protein.

**DISCUSSION**

These results show that the unit gravity sedimentation technique can provide significant enrichments of sub-populations of a total cell suspension of cerebellar cells prepared by the controlled enzymatic isolation method described in the previous chapter. Peaks of enrichment have been defined on the basis of size, and a set of criteria established which allows reproducible identification of these fractions from one experiment to the next. The Coulter analysis, which is the basis of this identification, can be swiftly performed and requires only small samples of the separated cell fractions. The peak fractions have been shown to be enriched in cells of distinct morphology, and a separation of replicating cells has been achieved. The separated fractions have been shown to be relatively undamaged by morphological and biochemical criteria. A summary of the results for each peak fraction follows.

**Fraction E (Diameter >14.5μm)**

Purkinje cells. The large size and structural features of these cells leaves little doubt that they are neurons. Many show morphological features characteristic of Purkinje cells at this stage
of development, however the large neurons of the deep cerebellar
nuclei and Golgi II neurons might also be expected to occur in this
fraction. Golgi II neurons represent only 10% of the Purkinje cell
numbers in situ (Eccles et al, 1967) while the large neurons of the
deep cerebellar nuclei are in a more advanced stage of differentiation
and are multipolar and therefore more likely to be lost during the
cell isolation procedure.

As many as 50% of the E peak cells are contaminating small cells,
however this represents a many-fold enrichment of E size-range cells
over the 1-2% seen in the total cell suspension. The preponderance
of large neurons in the E peak is over 80% if expressed on the basis
of tissue mass, and therefore this fraction is promising as a starting
material for biochemical studies on Purkinje neurons at this stage
of development.

Fraction D (Diameter 10-14.5μm)

This was the most heterogeneous fraction. It contains cells of
several different morphological appearances and includes the larger
of the proliferating cells which are most strongly represented in
C. Unlike the other fractions it was not enriched in any one particular
cell type by the criteria used. However D remains a fraction of
interest, for it is in this size range that the inhibitory interneurons,
known as basket and stellate cells, are to be expected. The largest
of the proliferating cells could quite possibly be not external
granule cells, but precursors of stellate or basket cells. It is
possible that further investigation with biochemical markers such as GABA uptake will reveal an enrichment of these cell types in this fraction.

Fraction C (8-10µm)

Dividing external granule neuroblasts and immature granule neurons. This fraction is particularly enriched in dividing cells as indicated by high levels of thymidine incorporation, and numbers of cells actually in mitosis. 35% of this peak is in S-phase or mitosis.

Fraction B (6.5-8µm)

Immature and premigratory granule neurons and possibly dividing external granule neuroblasts in the G-I phase of the cell cycle. The B peak has the highest concentration of cells of any fraction from the chamber sedimentation; B is also the most common size range in total cell suspensions from older animals (>6 days postnatal, see previous chapter). With the exception of their size, these cells are morphologically similar to the non-mitotic cells in C. However they differ by their low levels of thymidine incorporation and the virtually complete absence of cells in mitosis.
Fractions B and C are seen as discrete cell species in the Coulter analysis rather than as different regions of a continuous range of cell size. This distinction is reflected in the marked biochemical differences found. It seems certain that C contains the dividing external granule neuroblasts which are in S phase. It may be that B consists of those cells from the same population which are in G-1 phase and therefore smaller (the mean volume of B cells is just over half that of C cells). This would agree with the conclusions of Barkley et al (1973) for unit gravity sedimentation of mouse cerebellar cells. However, there is some evidence to suggest that B consists primarily of the immature or premigratory granule neurons which form a substantial layer beneath the dividing external granule layer, particularly in older animals (Altman, 1972b). This would explain the notable transition in preponderance of numbers in the TCS from C size cells to B size cells with increasing age of animals. Figure 2.1 in the previous chapter shows that in a 6-day animal C cells account for 41% of the TCS while B cells are only 30%. By 12 days of age C cells are 29% and B cells are 45%.

As detailed in Chapter 5, cell cultures of Fractions B and C are remarkable mainly for their similarity, and this supports the view that these fractions are of very closely related cell populations and that the sharp biochemical distinctions observed are a reflection of different stages of the cell cycle. Cultures of Fractions B, C and D contain some astroglia with the capacity to divide, these will have contributed to the labelled thymidine uptake seen here in vivo and in vitro. However, variations in the small
proportion of glia between cultures of B and C cannot account for the large observed differences in the number of mitotic cells.

**Fraction Bp (Diameter 6.5-10μm)**

Astroglia-like cells. The morphological difference between these cells and the similarly-sized C and B cells is very great, and Bp cells strongly resemble cerebellar astroglia. It is quite conceivable that glia have a sufficiently different density from neurons to cause the 'paradoxical' migration shown by Bp cells. After all, most bulk separation methods for neurons and glia are by gradient centrifugations based on density differences between the cells.

However, several inconclusive strands of evidence point to an alternative possibility that these are damaged cells. The Bp cells migrate on the chamber adjacent to the A peak of damaged cells and cell debris. They show a higher proportion of damaged cells in electron micrographs, although they continue to exclude trypan blue. They show very little *in vivo* or *in vitro* incorporation of labelled thymidine, even though most glia are dividing actively at this stage of development. The Bp cells show a very low rate of protein synthesis when compared with all the other cell fractions; however it is believed that the glial protein synthesis rate is quite low, at least in the adult brain (Droz and Koenig, 1970). In addition, Malcolm East in our laboratory, has shown that the proportion of Bp rises markedly when the cells are incubated at
37 °C for 1 hour prior to separation (unpublished).

Clearly a definite identification of these cells must await further studies, although some information is provided by the culture work detailed in Chapter 5.

Fraction A (5.5-6.5μm)

Debris, free nuclei, and heavily damaged cells. The identification of this fraction is quite conclusive, it represents the upper size range of the small subcellular material which does not sediment significantly from the cell loading layer.

It has been shown that considerable enrichments of sub-populations of cerebellar cells can be achieved by unit gravity sedimentation. However the question of yield is also important when assessing the usefulness of a cell separation technique. Yields of cerebellar DNA isolated into the total cell suspension range from 30% to 50%. About 90% of the cells loaded onto the chamber gradients are recovered in the fractions collected. However many fractions must be discarded if only the peaks of enrichment of cell classes A - E are to be used. Typically, a sedimentation of 185 x 10^6 cerebellar cells from 7 day rats, will give 15-20 x 10^6 cells in the B peak, 10-15 x 10^6 cells in the C peak and 1-1.5 x 10^6 cells in the E peak. These quantities are quite practical for biochemical or cell culture studies.
The application of Coulter size analysis to the separated cell fractions produced by unit gravity sedimentation has proved to be important in two respects. Firstly it has allowed the definition of a number of size-based cell classes which have proved to be distinguishable on the basis of morphological and other criteria. Secondly, it provides a quick, reliable means for the routine monitoring of fractions for the cell size peaks. This is vital if unit gravity sedimentation is to become a practical routine preparation procedure for separated cells, as the alternative of visually monitoring chamber fractions by phase contrast microscopy is both highly laborious and very unreliable.

Unit gravity sedimentation, allied to Coulter size analysis, is a practical, reproducible, technique for the preparation of enriched fractions from the total mixed suspension of cerebellar cells. The damage to the cells during the separation seems to be negligible by morphological and biochemical criteria. It remains to further characterise the fractions by means of available markers for cerebellar cell identity and to discover if they fulfil their promise as a defined starting material of restricted cell type for cerebellar cell culture.
It is customary to cite the work of Harrison (1910) on the growth of frog neural epithelium in a plasma clot as the first instance of both tissue culture in general and nervous system culture in particular. Certainly it has been amply demonstrated over the past several years that nervous tissue can develop or maintain high degrees of differentiation in culture. Essentially every region of the neuraxis has been successfully grown in some form of culture and neural tissue from invertebrates, amphibians, birds, rodents, and humans has proven suitable for in vitro work (Murray, 1971; Nelson, 1975). Cultures of the nervous system have been extensively studied by the methods of the neurophysiologist, anatomist and biochemist. However the great promise that simplified culture systems hold for the biochemist has not yet been fully exploited. A partial review of the culture literature may reveal some possible reasons for the current limitations of biochemical studies on neural cultures, particularly when compared with the extensive electrophysiological, pharmacological and ultrastructural results which have been obtained.

The type of question that can be asked of a culture system
depends upon the type of culture used. Explant cultures consist of small (approximately 1 mm³) fragments of tissue which when maintained in a suitable medium exhibit a relatively high degree of functional and anatomical integrity and may rather faithfully reflect some organisational features of the intact nervous system. On the other hand, this complexity precludes morphological clarity so that experimental rigour is sacrificed to some degree. Explant culture studies have provided much useful information on the development and details of organisation of many regions of the nervous system, including the cerebellum. However, these were electrophysiological and ultrastructural investigations, explant cultures offer few advantages for biochemical work due to their complexity and the small amount of tissue available. Recently "in vivo explant cultures" have been prepared by implanting small pieces of brain in the anterior chamber of the eye where vascularisation of the explant occurs (e.g. Ljungdahl et al, 1973). This system involves better preservation of tissue integrity and has considerable potential for the study of developmental specificity, as does the related technique of transplanting pieces of tissue into different areas of the brain (Das, 1975). Both systems have little to offer the biochemist.

A less organised and more homogeneous system is provided by dissociated cell cultures, prepared by rigorously disrupting all intercellular contacts in immature neural tissues usually by a combination of enzymatic and mechanical procedures (Paul, 1973). Dissociated cells may be grown in shaker flasks when they form
re-aggregates, or as monolayers on a flat surface. Many features of normal neuronal development, including synapse formation, are reproduced in these cultures although clearly the spatial organisation of the cells is quite different from \textit{in vivo}. Dissociated cultures usually contain several cell types, but are reproducible and available in some quantity, depending on the source of material. They are therefore amenable to biochemical techniques but present some problems of interpretation.

Truly homogeneous cultures are formed by continuous cell lines of transformed or neoplastic nervous system cells, which while retaining their capacity for cell division, are also capable of expressing a number of differentiated characteristics under appropriate culture conditions. These cell lines have clear advantages for biochemical analysis, but differ from the intact brain more markedly than do cell cultures of normal material (Sato, 1973). For instance, the much-studied neuroblastoma cell line cannot form synapses, although a hybrid neuroblastoma/glioma line is now reported to form synapses in culture (Nelson et al, 1976). The trade-offs that must be made between analytical advantages and potential or actual sacrifice of normal biology can best be discussed with regard to specific experimental situations (see Giller et al, 1975).

**EXPLANT CULTURES**

The culture of tissue explants from the nervous system (often
simply referred to as "tissue culture") has a long and rich history. It has been reviewed in recent times by Murray (1971), Nelson (1975), and in an elegant monograph by Crain (1976). The most extensively studied system is explants of whole sensory or sympathetic ganglia from the embryonic chick or, less commonly, rat (this could also be regarded as a form of organ culture). These ganglia can be obtained relatively easily and react favourably to the culture environment. The demonstration of myelin formation in cultures of chick dorsal root ganglia (DRG) by Peterson and Murray (1955) was an early and compelling piece of evidence that complex neural development could occur in vitro. Crain (1956) showed by intracellular recording that similar cultures exhibited quite normal electrical properties.

Culture of these ganglia has been facilitated by the discovery of nerve growth factor (NGF), a potent protein factor which induces rapid process outgrowth from certain neuronal cells including sympathetic ganglion neurons and one class of DRG neuron. Indeed ganglion cultures have been commonly used as a bio-assay for NGF (reviewed by Levi-Montalcini and Angeletti, 1968). Roisen et al (1970), using a quantitative measure of neurite outgrowth have claimed that cyclic AMP can mimic NGF action, and may mediate some of NGF action. However, other workers (Frazier et al 1973) dissented and claimed separate actions of cyclic AMP and NGF on process extension. The effects of NGF are further discussed below.

Ganglion explants have also been used in the exploration of the mechanisms of process outgrowth, and a large proportion of our
knowledge of process extension and of the activity of the growth cone comes from this work. Nakai (1960, Nakai and Kawasaki, 1959) extended the classic observations of Weiss (1934) on the nature of neurite extension emphasising the dynamic character of growth cone behaviour and the differential response of the "palpating" exploring tip of the neurite to different objects. Nakai saw a general initial adhesiveness modulated by specificity in the formation of permanent attachments and a striking propensity for "fasciculation" or bundle formation of growing processes was noticed. More recently, Yamada, Spooner and Wessells (1970) have described the structure of the growth cone in considerable detail and have shown that the structure and motility of the cone is dependent on the existence of a polygonal meshwork of 4-6nm filaments which is sensitive to cytochalasin B, implying an actin/myosin based structure.

The spinal cord may also be maintained in explant cultures, typically an entire cross-section or semi-section is explanted; spinal cord structure is largely retained in these explants, as beautifully shown by Sobkowicz et al (1968). Peterson, Crain and Murray are responsible for much of the work in this field. They reported (Peterson et al, 1965) studies of electrical properties and morphological changes, including myelination, in spinal cord explants. Myelination began two to three weeks after explantation and was of the central oligodendroglial type within the explant. Myelination of the outgrowing processes was of the peripheral Schwann cell type if the meninges, a source of Schwann cells, were
Included. However, absence of meninges resulted in central-type myelination of the outgrowth. Complex bioelectric activities could be recorded, however the extracellular techniques used prevented clear delineation in the cellular mechanisms involved.

In 1967, Bunge, Bunge and Peterson showed that synapses had developed in vitro in explants from young rat fetuses where there are no synapses yet formed in the spinal cord. James and Tresman (1968) showed that neuromuscular junctions could develop in co-cultures of spinal cord explants with immature muscle tissue. Peterson and Crain (1970) made a detailed study of a similar system and found a notable lack of species specificity in the formation of junctions between rat and mouse tissue. An important role for Schwann cells in the maturation of motor end plates was indicated in this work.

Neuromuscular synapses formed in apparently normal fashion during chronic blockade of their function by curare or hemicholinium (Crain and Peterson, 1971). This important observation has also been made in other parts of the nervous system. For instance, Crain, Bornstein and Peterson (1968) showed that the synaptic maturation (monitored electrically) of cerebral cortex explants was not prevented by chronic treatment with Xylocaine or a high magnesium ion concentration, both of which effectively blocked synaptic transmission. Model et al (1971) made an ultrastructural study of the same phenomenon. This is strong evidence that the functional interactions of neurons do not play a major part in the
development of the synaptic networks of the nervous system, although does not exclude the modulation of those networks by function which may be the basis of the brain plasticity.

Cerebellar Explants

The cerebellum is the brain area which has been most extensively studied in explant culture and has been recently reviewed (Seil, 1979). Pomerat and Costero (1956) found Purkinje cells to be clearly identifiable by their characteristic morphology and tendency to remain within the explant. The granule cells were typically migratory and similar to oligodendrocytes although the latter exhibited pulsations (Lumsden and Pomerat, 1951). More recently Wolf has focussed in detail on the morphology, synaptic connections and migration of granule cells (Wolf and Dubois-Dalcq, 1970; Wolf, 1970).

Allerand (1971) made a highly detailed study of cerebellar explants by light microscopy using Holmes' reduced silver method and comparing with Cajal's descriptions of cerebellar cell morphology when stained by a similar method. Using this technique she was able to identify several neuronal types, in particular granule cells, with more certainty than had previous workers. She described the formation of an internal granular layer during the course of maturation in vitro, which was correctly positioned, although of reduced size compared with in situ. Granule cells were also seen to actively migrate into the outgrowth zone of the explant and cells of characteristic morphology, including a
bifurcatory axon were described. During her discussion of this work, Allerand emphasised the importance of recognising transitional states of development as such, and not as abnormal developments peculiar to the culture situation. She took as an example the persistence in vitro of Purkinje cells with multiple primary dendritic processes which are a normal neonatal developmental phase but which had been described by Wolf and Holden (1969) as a discrepancy between in vitro and in vivo behaviour.

Several groups have made the apparently surprising observation of mossy fibre and parallel fibre terminals in developing explants of cerebellum (Kim, 1971; Chesanow et al 1970; Wolf and Dubois-Dalcq, 1970 and Hendelman; 1975). This paradox may have been resolved by Privat and Drian (1975) who clearly showed the formation of glomeruli, the mossy fibre/granule dendrite synapses, in co-cultures of explanted cerebellum with a portion of the superior vestibular nucleus. This nucleus contains some of the cells of origin of mossy fibres. However, cultures of cerebellum alone did not develop glomeruli. Seil and Herndon (1970) also found no mossy or climbing fibres in their preparation of cerebellar explants. Privat and Drian suggest that the discrepancy may be due to different methods of tissue removal and note that both Kim, and Wolf and Dubois-Dalcq mention that their tissue may include varying amounts of vestibular nuclei.

Glomerulus formation under the conditions of Privat and Drian is interesting. Mossy fibres were able to find their way through
a disorganised tissue to reach their granule cell targets. The basic and very characteristic structure of the glomerulus was then built up indicating a pre-eminence in this process of factors intrinsic to these cells. The involvement of the postsynaptic structure, in this case the granule cell, in the elaboration of this complex synapse is indicated by the formation in vivo of very different synapses when mossy fibre collaterals synapse in the cerebellar nuclei.

Extensive electro-physiological studies on cerebellar explants have been made by the group of Gähwiler (e.g. Gähwiler et al., 1972) showing complex and varied patterns of firing. Synaptic relationships between cells were found and the effects of ions and drugs were investigated (Gähwiler et al., 1973). More recently, Gähwiler has shown (1978) that when cerebellum is cultured with inferior olive complex spikes can be recorded from Purkinje cells which are similar to climbing fibre responses. The future for explant cultures may lie in work such as this. There are many experiments to investigate the specificity of the climbing fibre connections and their developmental profile which can be performed in such cultures. The climbing fibre/Purkinje cell synapse will not be available for study in dissociated culture unless or until great advances are made. Many experiments can (and therefore should) be performed in vivo, particularly as climbing fibres can be specifically destroyed by the nicotinamide antagonist 3-acetyl pyridine (Desclin and Escubi, 1974). However the availability of a specific culture system such as Gähwiler's
permits a much wider range of experimental design.

With the possible exception of a specific system such as that described explant cultures have little to offer a biochemical approach to development. Several groups have looked at the development of normal properties in explants of brain areas including biochemical parameters such as enzyme development (Lehrer et al, 1970) and amino acid transmitter uptake (Hösli et al, 1973). Serum deprivation has been shown to retard myelination and synapse formation in cerebellum explants, and this was suggested as an \textit{in vitro} model for malnutrition (Kim and Pleasure, 1978). A more clinically-oriented (and more interpretable) culture model is seen in the observation that sera from patients with multiple sclerosis and from animals with experimental allergic encephalo-myelitis caused demyelination in CNS explants (Bornstein and Appel, 1965). Oligodendrocytes were markedly reduced in number (Raine and Bornstein, 1971), but EAE serum did not have an effect on peripheral (Schwann cell) myelin (Bornstein and Iwanami, 1971)

\textbf{RE-AGGREGATING CULTURES}

Culture of re-aggregates of dissociated cells is a system which currently seems somewhat out of vogue. At the time of a 1973 review (Seeds, 1973) there was considerable interest and suggestions that cells differentiated more completely in a three-dimensional arrangement than when plated in a monolayer.
Seeds and co-workers have shown the development of synapses (Seeds and Vatter, 1971) in reaggregates of mechanically and trypsin dissociated cells from whole rat brains, and concentrated particularly on maturation by several biochemical criteria (Seeds, 1971). Changes in cell surface properties related to cell aggregation were studied as a function of developmental stage by Kleinschuster and Moscona (1972). Plant lectins were used as probes for the carbohydrate moieties of surface molecules. Garber and Moscona (1972a) showed patterns of reaggregation that were specific for particular brain regions, the size of the aggregate depending on the degree of homology of the constituent cells. Large aggregates were formed by cells from the same brain region, smaller, less cohesive aggregates from cells of different brain regions. Brain cells intermingled with non-neural cells completely 'sorted out' during aggregate formation. They went on (1972b) to show that a factor secreted into the medium by cultures of mouse cerebrum promoted aggregation of embryonic mouse cerebral cells, but not of cells from other brain regions, however there was an effect on cerebral cells from the embryonic chick. Garber and Moscona discuss these results in terms of specific recognition sites on cells from a particular brain region, which have been conserved through evolution.

A similar interest in specific patterns of aggregation and possible factors of developmental significance is to be seen in the work of Roth on aggregates of frog retina and tectum (e.g. Barbera et al, 1973) and of Lilien on chick neural retina cells, and the
factors required for their aggregation (Rutz and Lilien, 1979).

Certainly the most remarkable report of histospecific patterns of cell aggregation is that of Delong and Sidman (1970) who claimed not only that a re-creation of region-specific features of cellular organisation occurred in aggregates of mouse cerebral cortex, hippocampus and cerebellum, but also that some aspects of the disorganisation found in vivo in the mutant mouse strain _reeler_ were reproduced in the aggregates. This result carried a double implication; that the aggregates were a good model to study the mutational defect, and that the 'sorting out' seen in aggregates of normal mouse was of relevance to the cellular organisational processes taking place in vivo. Unfortunately there has been no repetition or extension of this work in the intervening years and this must lead to a questioning of the original interpretation. It is possible that the light microscopic histological techniques used were not sensitive enough to eliminate a cell sorting phenomenon due to non-specific factors such as differences in viability.

Another strand of work on aggregate cultures is the examination of biochemical differentiation. The transition from embryonic to adult isozymes of glycerol phosphate dehydrogenase has been studied in reaggregating cultures of mouse brain (Kozak, 1977). Moscona has suggested, from work on chick retina, that some aspects of biochemical differentiation can occur _in vitro_ in explants or reaggregates but not in monolayer cultures (Moscona et al, 1972). In reaggregating cultures it is difficult to determine the cell
composition, however proliferation of non-neuronal cells is known to be promoted in monolayer cultures (Varon, 1975b) and this may be behind many of the apparently lower levels of transmitter-related enzymes found in early comparisons of monolayer with aggregate cultures. The enzyme producing cells themselves may have developed in monolayer in the same direction as they did in aggregates, but have been diluted by other cells. The composition of aggregate cultures may also be subject to complex changes with time which affect the interpretation of biochemical data. Extensive gliosis and neuronal death was found in re-aggregating cultures of mouse cerebellum and not in similar cultures of cerebral cortex (Kozak et al, 1978).

Re-aggregating cultures retain all the disadvantages consequent upon rigorous disaggregation techniques while sacrificing the morphological clarity of monolayers. It is possible that studies of the aggregation process itself may be of great importance, it seems unlikely however that reaggregates are an optimal system for the study of differentiation, despite the earlier suggestions of workers in this field.

MONOLAYER CULTURES OF THE PERIPHERAL NERVOUS SYSTEM

As was the case with explant cultures, the dorsal root sensory ganglion (DRG) of the chick embryo has proven a favourable and much-used preparation in early work exploring the possibilities of dissociated cultures. Many different procedures for cell dissociation
have been used and cells have been grown in various media on various substrates. However, some features have now become fairly universal. Bornstein (1958) showed that substrates coated with reconstituted rat-tail collagen were advantageous for explants of nervous tissue and this has generally been found true for dissociated cultures although preferable treatments have more recently become available (see below).

Typical dissociation procedures seem, when compared with the method described in Chapter 2, to be rather harsh. For instance, Scott, Engelbert and Fisher (1969) incubated isolated DRG's with 0.25% trypsin in a simple salt solution for 45-60 min. at 37 °C, followed by trituration and plating (i.e. a 10-fold greater trypsin concentration, for 3-4 times longer, with no supporting BSA and no trypsin inhibitor). This group first made the important observation that elevated K⁺ levels promoted neuronal survival in some culture conditions. They found that 10-fold more DRG neurons survived when the cells were plated in 40 mM K⁺ ion compared with normal (6.8 mM) K⁺ concentration (Scott and Fisher, 1970; 1971).

Surface cultures of dissociated DRG cells are by no means necessarily true monolayers. Non-neuronal cells characteristically cover the surface of the dish (Scott et al, 1969). Neurons rest on top with their processes penetrating into the layer of non-neuronal cells and connective tissue. Early work showed that extensive branched networks of neuronal processes were readily produced in DRG cultures (Nakai, 1956), recurrent collateral axons frequently approaching their
cell body of origin. Rotation of nuclei was measured in detail and bidirectional movements of vacuoles and mitochondria in the neurites were described. Lodin et al (1970) also described nuclear rotation in dissociated chick DRG cells and emphasised the making and breaking of cell-cell contacts, pointing out the reassociation that takes place between neuronal and satellite cells. A close relationship between even single outgrowing fibres and supporting cells was observed and various forms of myelination were described (Lodin et al, 1973).

Miller et al (1970) saw synaptic profiles in dissociated chick DRG cultures, a surprising finding as spinal cord cells are presumably absent and they were always presumed to be the source of synapses seen in vivo on afferent sensory axons in the spinal cord. Possibly afferent fibres have at least the capability of synapsing with each other. In a carefully reasoned summary of the specificity of synapse formation seen in culture, Bunge (1975a) cites this brief report of Miller et al as the only example of synapse formation in culture for which an in vivo counterpart cannot be found. The synaptic nature of the EM profiles shown cannot be questioned. However, this result must be set against a lack of electrophysiological evidence for synapses (Varon and Raiborn, 1971; Okun, 1972) and the wealth of morphological observations by the Bunge's and co-workers on rat DRG cultures which have failed to reveal any synapses (e.g. Bunge et al, 1967).

**Separated cell cultures of peripheral neurons**

It would be of great advantage in the study of the interactions
of DRG neurons with other cells, to be able to culture the neurons in the absence of 'supporting' non-neuronal cells. In the case of the sympathetic ganglion cells described below, such a method became available early (Bray, 1970) and has contributed greatly to the work in this area. An early method for DRG cultures involved the free-hand dissection of neurons and their subsequent culture (Sensenbrenner et al, 1969, Shahar and Saar, 1970). This clearly put limits on the amount of material in culture; procedures using sieving were less successful in eliminating non-neuronal cells (Sensenbrenner et al, 1969). A histochemical study of cultured DRG neurons isolated by free hand dissection indicated that acetylcholinesterase, monoamine oxidase, glutamate dehydrogenase and other enzymes persisted for several days in culture, NGF prolonged this period (Ciesielski-Treska et al, 1970). Other methods have depended on the differential adhesion of neurons and non-neuronal cells to collagen surfaces (Varon and Raiborn, 1972), glass beads (Okun, 1972, Okun et al, 1972), or collagen surfaces during intermittent agitation (McCarthy and Partlow, 1976a). By these means, purifications of neurons from 90% to '>99%' have been achieved for dorsal root and sympathetic ganglion cells from the chick, mouse and rat.

Extensive networks of cells and processes formed in the virtual absence of non-neuronal cells were studied electrically by Okun (1972) who found rather complex patterns of responses to stimuli, although clearly substantially less complex than those found in dissociated CNS cultures (see below). The complex responses evoked by single
stimulus pulses suggested that many processes, particularly in older cultures, contained more than one single active fibre, some being 'driven' by others. However, there was no conclusive evidence for synaptic connections between fibres. Some neurons were found to be capable of developing electrically active processes in the absence of contacts with any other cells. Processes were active very soon after outgrowth, although a region of immaturity just behind the growing tip was indicated, the duration of such immaturity being no greater than 20 hours.

A considerable extension of the work described in the section on explants on the structure and functioning of the growth cone has been achieved in dissociated cultures, mainly of chick sympathetic ganglia. Particularly important is the work of Bray (1970, 1973a,b) who studied the growth patterns of single isolated neurons in culture. He showed that membrane material was added at the growing tip rather than more proximally (1970), and that the shape of the fibre network was largely a product of the activities of the growth cones (1973a). He went on to propose a detailed model of the apparatus responsible for the striking motility of the growth cone, and similar structures (1973b). Yamada, Spooner and Wessells (1971) made a detailed ultrastructural analysis of the growth cone indicating that microtubules essentially do not enter the growth cone and that there is an extensive 4-5nm microfilamentous network in the growth cone. M. Bunge has studied cells isolated in culture by Bray's method at the ultrastructural level (Bunge, 1973). A contractile protein, possibly identical
with actin was found to be produced by growing DRG cells (Fine and Bray, 1971) and it is now generally accepted that actin is organised to form the network of microfilaments seen in the growth cone (e.g. Luduena and Wessells, 1973).

The effects of nerve growth factor

Most neurons from the DRG and practically all from sympathetic ganglion will die in dissociated culture unless they are supplied with NGF. Work with this growth factor in dissociated cultures has shed some light on its possible mode of action in vivo. Dependence on NGF of these neurons in vivo cannot be demonstrated unless all possible sources of the factor can be shut down. Immunosympathectomy, the destruction of sympathetic neurons by administration of anti-NGF, indicates that such a dependence exists, however this effect could result from complement-mediated cell lysis rather than NGF deprivation (Steiner and Schonbaum, 1972). All available evidence indicates that NGF does not change the course of differentiation but only affects the rate of expression of normal differentiated function. It appears to be necessary for neuronal performance over a much longer span of the susceptible cell's life history than would a differentiation factor and it has thus been described as a modulation factor (Varon, 1975a). These properties of NGF can be contrasted with the factor described in sympathetic ganglia by Patterson and Chun (see below) which, during a short sensitive period, induces a qualitative change in the course of development, rather than a quantitative change in its timing.

The postnatal mouse DRG is not dependent on NGF when cultured
as an intact explant. However dissociated neurons from this ganglion did require NGF in cultures where there were few surviving ganglionic glial cells (Varon et al, 1973). When the cultures were supplemented with DRG non-neuronal cells, the requirement for NGF disappeared and maximal neuronal performance could still be elicited. Ganglionic non-neuronal cells could also substitute for NGF in cultures of chick DRG and sympathetic ganglion, the traditional NGF target tissues (Burnham et al, 1972). Species and age-homologous ganglionic non-neurons, identified as mainly glia, were found to be the most competent in supporting neuronal performance, the effective agent(s) appear to be released to the medium or culture surface (Varon et al, 1974a). The support of neurons by ganglionic glia is blocked by an antibody to mouse submaxillary beta-NGF (Varon et al, 1974b), encouraging a view of glia as the endogenous supplier to the ganglionic neurons of a factor which is functionally and antigenically similar to NGF. However an alternative possibility yet to be ruled out is that the glia act by capturing an exogenous NGF-like precursor which they activate and deliver to their accompanying neurons. The species and age selectivity of the effect may be due to minor differences in the NGF-like factor produced, or to different modes of delivery of an identical factor.

NGF action within the CNS is indicated by accelerated regeneration of catecholamine-containing axons after lesions of medial forebrain bundle in adult rat on intracerebral or intraventricular administration of NGF (Bjorklund and Stenevi, 1972). Conversely, this regeneration is retarded by an antiserum to NGF,
indicating there may be a source of NGF intrinsic to the CNS, as NGF cannot pass the blood brain barrier (Bjerre et al, 1974). Behavioural recovery after hypothalamic lesions is also reportedly enhanced by intracerebral NGF injection (Berger et al, 1973). NGF has been reported to enhance development of catecholamine-containing neurons in explant cultures from early head neural crest (Bjerre and Bjorklund, 1973), but there have been few other reports of NGF action in CNS cultures, and its involvement at this level must remain in question until specific effects on identified neurons in vitro have been demonstrated.

Observation of a cellular response to NGF requires not only a programmed sensitivity of the potential target neuron but also an experimental situation in which the cell is performing below its maximal capacity so that NGF may be seen to enhance performance. Thus the response of postnatal mouse DRG neurons to NGF could not be seen until they were deprived of their glial support. However, in some cases, the presence of non-neuronal cells, although non-NGF producers, is required for the NGF response, placing a third restriction on the experiment. The interaction of NGF with the target cell surface and possible further sites of action within the cell have been extensively studied in culture (see Varon, 1975a; Varon and Bunge, 1978) and will not be further considered here. An ionic mechanism for short-latency NGF action has recently been proposed (Varon and Skaper, 1980).

Mitogenic effects of neurons on non-neuronal cells

Another example of an important interaction between neurons and glia which has been clarified using cultures of the peripheral nervous system is the mitogenic effect of axons on Schwann cells. Methods for the culture of Schwann cells alone have been reported (Wood and
Bunge, 1975; Wood, 1976). These depend on the proliferation of Schwann cells among the outgrowth of an explanted DRG, the ganglion can then be excised, the axons die back and the remaining bed of Schwann cells will survive for many months, but shows no cell division. If bare DRG axons are allowed to grow into such a quiescent Schwann cell bed there is active mitosis (³H-Thymidine labelling) in all areas contacted by axons, but not in adjoining areas. It appears that direct contact between axon and Schwann cell is required for this mitogenic effect. In time the Schwann cells will proliferate and myelinate the new axons. Similar information has also been obtained in a chick system (Varon, 1977). Using a different system based on initial separation of autonomic neurons from their associated non-neuronal cells by a differential adhesion method, it has also been shown that the presence of neurons increases thymidine incorporation into non-neuronal cells (predominantly Schwann cells) by 230-370% (McCarthy and Partlow, 1976 a,b). If the two cell populations were allowed to communicate only via the medium, then the effect did not occur, indicating that direct cell-cell contact is involved rather than a soluble factor such as NGF, or the factor of Patterson and Chun (see below). There have been reports that the particulate fraction of homogenates of neurons will stimulate thymidine incorporation into non-neuronal cells (Hanson and Partlow, 1978; Salzer, Glazer and Bunge, 1977). A recent study suggests that the mitogenic effects of cell sonicates, and of intact neurons are mediated by different mechanisms (Hanson and Partlow, 1980).

**Cell cultures of sympathetic ganglia**

It is probably in culture of the autonomic nervous system that the greatest progress has been made in biochemical work on elucidating
the control of neuronal development by the cellular environment. Most work has been done on long-term cultures of dissociated sympathetic neurons from the superior cervical ganglion (SCG) of perinatal rat. The principal neurons of this ganglion are 95% adrenergic and 5% cholinergic (Yamauchi et al, 1973). Mitosis of neuronal precursors is essentially completed by the first postnatal day in SCG although the neonatal neurons are relatively immature. Their survival in the first few weeks of culture is dependent upon the presence of nerve growth factor in the medium.

It was initially found that SCG neurons retained their adrenergic properties in cell culture, including catecholamine synthesis and accumulation (Mains and Patterson, 1973a; Rees and Bunge, 1974). They were used for extensive studies of catecholamine metabolism and its maturation with age in culture (Mains and Patterson, 1973b,c). However these neurons, taken from a predominantly adrenergic sympathetic ganglion were subsequently shown to be capable of making cholinergic synapses with one another (O'Lague et al, 1974; Ko et al, 1976) and with skeletal muscle in culture (Nurse and O'Lague, 1975). This was an unexpected observation and prompted a series of studies which have excluded several possible explanations. It has now become clear that the same neurons are capable of expressing either adrenergic or cholinergic properties under different conditions and some of the factors controlling this crucial aspect of neuronal character have been elucidated.

Mitosis of neuronal precursors is essentially completed in the
SCG by the first postnatal day. Further neuroblast cell division has not been observed in these (or any) cultures, and the total number of neurons does not change appreciably with time (Johnson et al, 1976; Lazarus et al, 1976). Changes in the proportion of functional neuronal types by selection from dividing sub-populations is therefore ruled out, as is selective cell death. The proportion of neurons with demonstrable cholinergic synaptic interactions was often very high (up to 70%) (O'Lague et al, 1974; Ko et al, 1976) and therefore could not be derived solely from the small number (5%) of cholinergic neurons present in the intact ganglion.

A more complex possibility is that the cholinergic neurons in these cultures arise from a population of undifferentiated cells, initially unrecognisable as neurons, whose rate of differentiation into cholinergic cells approximately equals the rate of adrenergic cell death. Two separate lines of experimental design have produced evidence arguing against this suggestion. Cytochemical studies of cultures undergoing a 'shift' from adrenergic to cholinergic function do not show a simple diminution in the numbers of adrenergic synapses accompanying a rise in the numbers of cholinergic synapses. Instead a transition state is seen, many synapses having a mixture of dense-cored (adrenergic) vesicles and clear (cholinergic) vesicles (Johnson et al, 1976). However the most convincing evidence for a transitional dual-function state comes from studies of single sympathetic neurons in culture (Landis, 1976; Furshpan et al, 1976). Single isolated SCG neurons were grown on small islands of previously
cultured rat heart cells. It was possible by physiological, pharmacological and cytochemical means to identify each neuron as adrenergic, cholinergic or dual-function. Stimulation of the dual function neurons first inhibited and then excited myocyte activity. Inhibition could be blocked by atropine and excitation by propanolol, a striking demonstration that a single neuron could in fact function with two neurotransmitters. Biochemical analyses of neurons grown in micro-cultures revealed that by four weeks in culture none of the neurons remained in a 'silent' or the remarkable dual function state seen at two weeks in culture (Reichardt and Patterson, 1977; Furshpan et al, 1976).

These experiments leave little doubt that a shift from adrenergic to cholinergic function can occur within individual neurons cultured from the rat SCG. The adrenergic neurons of this ganglion have not been shown in vivo to develop cholinergic characteristics when isolated from their preganglionic synaptic input or their visceral targets. Nor have the sympathetic fibres from adult mammals been observed to synapse on skeletal muscle in vivo. These neurons most readily developed cholinergic mechanisms when cultured during the first days after birth, at the time when sympathetic fibres are beginning to interact with their peripheral targets such as iris smooth muscle and pineal gland (Iversen et al, 1967). These observations led Bunge, Johnson and Ross (1978 and Ross et al, 1977) to speculate that during this early postnatal period a "specifying" signal from the adrenergic targets causes the sympathetic ganglion cells to be committed to adrenergic function.
Support for this sort of control is provided by the elegant embryological experiments of Le Douarin and her associates (1974, 1975) who have transplanted areas of quail neural crest into chick embryos where the quail cells remained cytologically identifiable. The general conclusions were that the neurotransmitter synthesised by a postmitotic sympathetic neuron is controlled by its immediate environment rather than being pre-programmed from a stage before its migration. However the role of selection could not be ruled out as the transplanted neuronal precursor cells continue to divide as they migrate. It is in the rigorous investigation of this sort of question where well-designed culture experiments can be of great use.

Much more specific information on the adrenergic to cholinergic switch seen in SCG neurons has come from the carefully controlled series of experiments of Patterson and Chun (1974, 1977a,b; Patterson, 1978). Conditions for growing SCG neurons in the presence or absence of non-neuronal ganglion cells had been established for some time (Bray, 1970, Mains and Patterson, 1973a). Cells grown in the absence of non-neuronal cells remained adrenergic, but in the presence of non-neuronal cells, high levels of acetylcholine synthesis and storage were found (Patterson and Chun, 1974). Furthermore, a medium conditioned by two days growth of non-neuronal cells could induce the cholinergic changes in the absence of the cells themselves (Patterson and Chun, 1977a). The effect was dose-dependent, larger amounts of conditioned medium induced greater amounts of acetylcholine synthesis and the formation of more cholinergic synapses; the
expression of adrenergic function fell off in a reciprocal fashion. Attempts to purify the macromolecular factor responsible for the effect have been reported to be under way (Patterson et al, 1978).

The results detailed above on age dependence of the switch to cholinergic expression found by Bunge and co-workers were closely paralleled in culture. The responsiveness of cells from perinatal animals to a 10 day pulse of conditioned medium declined rapidly with time in culture reaching a very low level when the pulse was applied between days 40 and 50 (Patterson, Chun and Reichardt, 1977; Patterson and Chun, 1977b). The effectiveness of non-neuronal cells from different parts of the body in inducing cholinergic characteristics in SCG neurons has been investigated (Ross and Bunge, 1976; Patterson and Chun, 1977a). There was a superficial correlation between the amount of cholinergic innervation a tissue receives and its effectiveness in this regard, skeletal muscle being very effective, heart muscle less so and liver least so. However several non-innervated types of cells were effective medium conditioners and, most remarkably, the non-neuronal cells from the sympathetic ganglion itself were effective. This is paradoxical as 95% of the SCG cells in vivo are adrenergic and yet they are surrounded by the ganglion non-neuronal cells. However the inducement of cholinergic character by conditioned medium is blocked via a calcium dependent mechanism by depolarisation of the SCG neurons (Walicke and Patterson, 1977). It is possible
that in vivo the interaction of incoming spinal cord fibres with
the SCG neurons during the first week after birth preserves them
in their prenatal adrenergic condition. Those 5% of the SCG
neurons destined to become cholinergic may acquire their
electrical input only after they have been influenced by non-neuronal
cells (Patterson, 1978). This sort of detailed, testable, hypothesis
would not have been possible without the results obtained from
careful culture work.

The progress of studies on the sympathetic neurons of the
rat spinal cervical ganglion has been considered in some detail.
Some general conclusions may be drawn from the relative success
of this work which are applicable to other tissue culture problems.
Firstly, the SCG was a very restricted and well understood source
of material; the transmitter(s) used by the cells were known
(95% adrenergic, 5% cholinergic) and it was possible for the
investigators to successfully assume that the neurons in culture
were of virtually a single type whose behaviour in vivo was known.
Secondly, several years ago a technique became available for
eliminating non-neuronal cells from the culture (Bray, 1970;
Mains and Patterson, 1973a) and culturing virtually pure cell cultures
of a single neuronal type. This depended on the availability of a
factor (NGF) which promoted neuronal growth without supporting cells.
The technique is based on culture in a bicarbonate-free medium
in air although these workers have now moved to the use of mitotic
poisons such as cytosine arabinoside to produce the same effect.

It is because the neurons in culture could be clearly related back to their counterparts in vivo that the physiological and cytological observations, particularly of Bunge and associates were meaningful. The early biochemical observations of Mains and Patterson (1973a,b,c) were assisted by the absence of non-neuronal cells. However the availability of virtually pure cultures was crucial to the later observations of Patterson and Chun (1977a,b). Clearly none of the results on the influence of different sorts of non-neuronal cells and of conditioned media on transmitter choice would have been possible if non-neuronal ganglion cells were present in the cultures.

The development of the intricate synaptic network of the adult nervous system probably involves innumerable "decisions" by cells which depend, as does this choice of transmitter, on cellular interactions. Several approaches will be required to disentangle this complex web of interactions, one will certainly be the study of cellular development in simplified and controlled culture environments.

**MONOLAYER CULTURE OF THE BRAIN**

Dissociated culture of the brain is the most recently developing aspect of nervous system culture. Its history now spans ten years and the number of groups involved is constantly rising. The work of about a dozen will be considered here.
The use of brain cell cultures had a rather clear inception. It began with the quite comprehensive report of Varon and Raiborn (1969) on the culture of cells from embryonic chick cerebral hemispheres. In this remarkable paper, the authors describe not only the successful culture of cerebral cells but also a partial fractionation of different cell types before culture. Cells were dissociated from cerebral hemispheres by a mechanical method using nylon mesh which the authors note, derives from that of Rose (1967). Cells were fractionated via a fairly complex procedure involving low-speed centrifugations on sucrose/BSA gradients, mild trypsinisation of some fractions and differential attachment of cells to glass surfaces. Three types of cell were recognised on the basis of their appearance. Type A were large neurons and could be prepared to 90% purity. Type B were probably smaller neurons and were not purified. Type C were of a flattened "epithelial" nature, could be prepared 100% pure and could proliferate in culture, unlike A or B.

The cultures were prepared in a clotted plasma layer and showed growth of fibres from the putative neurons and apparent formation of interacting networks between cells. In these fairly undeveloped culture conditions, A and B cells began to die out after 4 - 7 days in culture and by day 7 proliferation of C cells took over even those cultures which had been 90% enriched in A. The identity of the C cells was discussed, they were thought to be probably of ependymal origin and similarities to cultured glial cells were noted. The authors made the quite reasonable claim that the A and C
Preparations represented the purest materials for biochemical analysis then reported from neural tissues.

Varon has drawn attention in this paper and elsewhere (Varon, 1975b) to the importance for the neurochemist of achieving homogeneous cultures of defined cell types as a way of resolving the extreme heterogeneity of most brain preparations. It is probably significant that this aspect has not been emphasised by those reviewers whose own experimental approaches are primarily electrophysiological (e.g. Crain, 1976) or morphological (e.g. Bunge, 1975a) where the method itself provides corroboration of cell identity. It is ironic that, although great advances in the long-term culture of brain cells have since been made, Varon and Raiborn (1969) is still the only report of an attempt to fractionate cells and prepare purified cultures of defined cell types from the brain. However the same authors and others (see above) went on to prepare virtually pure cultures of neurons from the peripheral nervous system.

Sensenbrenner and co-workers cultivated mechanically dissociated cells from embryonic chick cerebral hemispheres in Rose chambers, (Sensenbrenner et al, 1971) and in flasks (Booher and Sensenbrenner, 1972). They observed that a collagen coating was advantageous for the growth of isolated neuronal cells, preventing clumping, for the first week in culture. After this a monolayer of "mesenchymal and glial" cells had formed upon which neurons differentiated and matured with long processes forming networks of fibres. Human and rat embryonic material was also cultured and in these cases a dissociation method using trypsin (0.25%) was found to be preferable.
Depending on the age of the embryos, neurons survived in cultures for more than six weeks. Addition of a crude brain extract (Sensenbrenner et al., 1972) was found to improve the apparent neuronal differentiation and some myelination was claimed in these cultures. Similar cultures were obtained from very young rat embryonic cerebral hemispheres (up to 9 embryonic days), but older embryos and newborn rats were not successfully cultured. Later work showed that dissociated nerve cells attached to a growing astrocyte layer in preference to fibroblasts, meningeal cells, or plastic and showed much more 'differentiation' as assessed by their phase contrast appearance (Sensenbrenner and Mandel, 1974). A synthetic tripeptide, Gly-His-Lys was found to partially substitute for serum during the first week in culture (Sensenbrenner et al., 1975).

An important step which moved beyond these morphological data was the demonstration by Grain and Bornstein (1971) in cultures of dissociated foetal mouse brain that extensive differentiation occurred as assessed by the more reliable criterion of electrophysiological measurement. The cultures were prepared on collagen coated cover slips in Maximow slide assemblies. Neurons tended to form aggregates of 2-200 cells. After 2-4 weeks in culture complex bioelectric patterns were detected in the larger aggregates including stereotyped oscillatory discharge patterns which had previously only been seen in well-organised undissociated CNS explants. These patterns and the pharmacological sensitivities found, indicated the presence of both excitatory and inhibitory synaptic mechanisms. The same culture system was observed by light and electron microscopy.
(Bornstein and Model, 1972); myelin development and synapse formation were shown to have occurred.

Biochemical studies in brain cultures

The Yavins and their co-workers are one of the most biochemically-oriented of groups working in this field and have made important technical advances. Their early papers describe the culture of rat embryonic cerebral hemispheres on plastic or collagen-coated surfaces. The cells aggregated together in suspension after 4-8 hours, then attached to the dish as aggregates and began to form neurites (Yavin and Menkes, 1973). These cultures were used for a series of biochemical studies of lipid metabolism (e.g. Yavin and Menkes, 1974a, b). However from the point of view of culture techniques, their most important contribution is the origination of the use of poly-L-lysine coated substrates for brain cell culture (Yavin and Yavin, 1974). 90% of cells plated attached to this surface within 20 minutes, and the formation of process was greatly facilitated. The rate of proliferation of background cells was also greatly reduced. They showed that D and L isomers and various degrees of polymerisation (M.W. 4,000 - 400,000) all had the same effect. Other poly-amino acids containing carboxyl or sulphhydryl sidechains were ineffective. Involvement of the amine side chain of lysine was further suggested by inhibition of attachment when the polylysine had been treated with dansyl chloride. Polylysine is now widely used as a culture substrate as it is more reproducible, simpler to use, and more effective in promoting initial attachment than collagen coating. This group have continued their lipid work with an extensive series of papers on the regulation of phospholipid metabolism in cultured cells from rat cerebral hemispheres (e.g. Yavin and Ziegler, 1977).
Schrier and co-workers have described biochemical 'differentiation' in cultures of whole newborn mouse brain and presented in some detail methods for assay of several neurotransmitter-linked enzymes in extracts of primary cultures (Wilson et al, 1972; Schrier et al, 1974). Although a more biochemical approach to cultures is welcome, it could be argued that these workers, in their early papers, put too little emphasis on determining the cellular content of their cultures by the admittedly inadequate methods then available. The few photographs shown in Wilson et al (1972) indicate an almost complete overgrowth by non-neuronal cells and the presence of very few apparent neurons. It is therefore not surprising that choline acetyltransferase (CAT) and glutamate decarboxylase (GAD) levels only attained 40-50% of those in freshly isolated cells, and only the level of GAD could be distinguished from that found in non-neuronal cell lines.

In a study on foetal rat brain cells of the effects of different media and cell plating density on 'growth' and marker-enzyme (CAT) development (Shapiro and Schrier, 1973) the authors expressed surprise that opposing effects of different media on the two parameters were seen. However as neurons do not divide in culture, it is to be expected that conditions which promote 'growth' measured by protein acquisition, which will mainly reflect cell division, will result in dilution of the CAT-producing cells. Schrier (1973) produced a several-fold increase in specific activity of CAT by sub-culturing foetal cultures of whole brain. Division of CAT-producing cells was strongly proposed although the alternative, and with hindsight
more likely explanation was mentioned in passing that in some way the sub-culturing procedure increased CAT expression in neurons while eliminating non-neuronal cells. The pictures shown support the latter interpretation.

In a following paper Schrier and Shapiro (1974) pioneer the use of fluoro-deoxyuridine as a mitotic inhibitor, an important contribution. They go on to present a detailed argument that neuronal (CAT producing) cells are dividing in their cultures. The argument is too complex to repeat here, but does not take account of the possibility that the presence or absence of overgrowing non-neuronal (glial) cells affects enzyme expression by the neurons. They regret the lack of a histochemical method for CAT to confirm their conclusion. However a light autoradiogram showing $^3$H-Thymidine incorporation into cells of neuronal morphology would have proved persuasive. To my knowledge, no such conclusive evidence of division of normal (i.e. non-transformed) neurons in culture has been presented by this or any other group.

The possible division of CAT-producing cells was mentioned again in Godfrey et al (1975), but this paper is of more interest for other reasons. It presents the first multi-disciplinary analysis of a single culture system. The development of mixed cell cultures from foetal rat brain is described by light microscopy (phase-contrast and silver staining), electrophysiology and biochemistry. With the use of fluorodeoxyuridine (FdU), morphologically defined neurons could be maintained for several weeks and showed a variety of neurite outgrowth arrangements. Complex electrical patterns were
recorded, including synaptic potentials; in some cases EPSP's and IPSP's were recorded from the same cell. Sensitivity to several neurotransmitters, particularly glycine and GABA, was revealed by iontophoresis. The optimum FdU schedule for these properties also proved to be optimum for development of CAT and AChE. The results and correlations shown are impressive and probably represent the most fully characterised culture system to that date. However, as the authors themselves state "A thorough analysis of neuronal cell types present in the cultures has not yet been performed". Indeed it is difficult to see how such an analysis could be performed on cultures of such mixed origin, presumably including neurons from all parts of the brain. Godfrey et al claim that the development and function of individual cells and small networks of synaptically linked cells can be analysed in great detail in these preparations. They go some way to achieving this goal. Nevertheless the study of such development in culture is of very limited usefulness if the results cannot be compared and contrasted with the behaviour of the appropriate cells in vivo.

Bonkowski and Dryden (1976) measured electrical responses of neonatal mouse brain cell cultures to transmitter substances added to the medium. Conventional depolarising responses were found to acetylcholine, histamine, dopamine and serotonin and hyperpolarising to GABA. However little response to glycine was found and it was suggested that the responses found by Godfrey et al may be a consequence of FdU action. They also found that some neurons clearly depolarised in response to noradrenaline which is generally regarded as a hyperpolarising inhibitory transmitter in the CNS,
chiefly due to its action on Purkinje cells. Once again this potentially important result is difficult to interpret due to lack of knowledge of the origin of the cells.

Cerebellar cell culture

Some groups have cultured more well-defined areas of the brain. The report of Nelson and Peacock (1973) on dissociated cultures of foetal mouse cerebellum is noteworthy in this regard and in that it is the first report of intracellular electrical recordings on dissociated brain cultures. Although neuronal survival rates were low, yielding a hundred or so neurons from a million cells seeded per dish, they were able to demonstrate characteristic EPSP’s and IPSP’s in these cells. They also carried out a rather elaborate analysis of the complex patterned activities generated by a group of four neurons in a one month old culture. This demonstrated the potential for the electrophysiologist of measuring interactive patterns in groups of cells connected by networks of neurites which can also be observed microscopically. The last sentence of this influential paper bears repeating here: "Analysis of the system clearly requires focussing on improved tissue culture methods for maintaining neuronal survival and for systematic identification of cell types by morphologic, biochemical and electrical means".

Perhaps the closest approach to this goal is to be found in the work of Lasher on dissociated cultures of two-day old rat cerebellum. Lasher and Zagon (1972) tentatively identify several types of cerebellar neurons in these cultures on the basis of silver staining and GABA intake. They introduce the use of high (24.5 mM) K⁺ levels to CNS cultures and show much improved survival at longer times in
culture with high $K^+$. In an important paper Lasher (1974) made a detailed autoradiographic study of $^3$H-GABA uptake into cells in these cultures. He documented the increasing complexity of process outgrowth from the labelled cells with time in culture, and showed that the onset of high-affinity GABA uptake occurred concomitant with the earliest stages of this differentiation. The labelled cells were mainly described as stellate neurons; the likely presence of basket cells is not discussed, although it had been mentioned in the earlier paper. A few labelled cells are described as Purkinje cells primarily because of their larger size (~20μm). More recently the virtual absence of Purkinje cells has been mentioned (Burry and Lasher, 1978 c). Lasher's identifications of cells are perhaps not as unambiguous as the 1974 paper suggests. This is nevertheless a close approach to the study of identified neuronal types of known brain origin, rather than vague classes such as 'neurons'.

Lasher (1975) went on to make a kinetic study of GABA uptake in culture. By contrasting normal cultures with neuron-depleted cultures he was able to give separate kinetic parameters for neuronal and non-neuronal uptake. The results indicated that previous estimated from slices, minces, or explant cultures were in error probably due to restricted access of the label to neurons. The author concluded that the GABA reuptake process is sufficiently powerful to re-accumulate all the GABA estimated to be released per nerve impulse within the duration of an IPSP.

An electron microscopic autoradiographic study of GABA uptake
Burry and Lasher, 1975) indicated that all parts of neuronal cells took up label to the same extent, and showed no obvious morphological difference between labelled and unlabelled synapses. However more recent quantitative work showed some differences in size and number of vesicles between $^3$H-GABA labelled synapses and the unlabelled synapses of, presumably, granule cells (Burry and Lasher, 1978 b).

The GABAergic synapses closely resembled those seen in vivo except that they did not develop the flattening of vesicles reported in adult cerebellar inhibitory synapses. The majority of GABAergic synapses were formed in a brief period between 10 and 14 days in vitro (Burry and Lasher, 1978c) which correlated well with the appearance of stellate cell synapses in vivo although their major postsynaptic partner, the Purkinje cell, is largely missing from these cultures.

The timing of stellate synapse formation was concluded to depend more on factors intrinsic to the cell than on such extrinsic factors as the presence of the normal postsynaptic cell. This also provides confirmation of the short time (~48 hr.) needed for complete synapse formation found in another culture system (Rees et al, 1976) and suggests that apparently slower changes reported from in vivo studies may in fact be a summation of many short-term changes in a heterogeneous population of synapses. The relative homogeneity in synapse and neuron population of this culture system compared to in vivo cerebellum was used to advantage in a detailed EM study of synapse morphology comparing conventional osmium-uranyl-lead staining with ethanolic phosphotungstic acid (Burry and Lasher, 1978a).

Messer (1977a) also used $^3$H-GABA autoradiography to mark cells
in dissociated cerebellar cultures from, in this case, the mouse. The main purpose was to show that the putative granule neurons did not take up GABA. The practical problems of culture media, batch testing of sera etc. were described at some length in this paper. It is possible that the apparent extreme sensitivity of Messer's cultures was due to the initial cell isolation method based on Barkley et al (1973) using 1.0% trypsin (cf. 0.025% in the method described in Chapter 2). Only 30% of preparations were reported to give 'good' results, neuron survival up to 3 weeks, although each complete cell preparation was consistent within itself, again suggesting that the problem lay at this stage.

Messer and Smith (1977) compared cultures of weaver and staggerer mutant mice with normal cultures. They showed no difference in survival of weaver granule cells in culture, which thus survived in vitro beyond the period of granule cell death in the intact weaver cerebellum, implying that cell death is not intrinsically programmed into weaver granule cells. This lends some support to the theory of members of the same department that the fault in the weaver lies in the interaction between granule cells and Bergman glial fibres (Rakic and Sidman, 1973a). Surprisingly, the granule cells of the staggerer mutant survived longer in culture than wild-type cells, and these results were discussed in terms of possible genetically-based differences in cell-cell adhesiveness. However a later paper (Messer, 1977b) mentions that the improvement in staggerer cell survival claimed by Messer and Smith was in fact an improvement from 30% to 80% in the proportion of cell preparations
giving optimum results. Improvements to culture surfaces (polylysine) and other conditions also eliminated this difference, which was again discussed as a meaningful consequence of mutation either directly in the granule cells or via an early interaction with another cell type in situ. In the opinion of this author the most likely explanation is that the rather harsh cell isolation procedure causes less damage to cells isolated from staggerer cerebellum as a non-specific consequence of the impaired organisation and inter-cellular connections in the mutant.

Another paper from the same department on dissociated cerebellum cultures takes a very different approach (Trenkner and Sidman, 1977). This is an elegant and detailed morphological study of the cell movements and associations seen in cultures of dissociated mouse cerebellar cells plated at high density in microwells. Cells were seen to aggregate after plating (on an uncoated plastic surface) and several cell types were identified on the basis of light and electron microscopy, including granule cells, stellate and/or basket cells, large neurons (possibly Purkinje cells), oligodendrocytes and astrocytes. Synapses formed after 72 hours in vitro, and specific patterns of cell interaction could be studied during the course of the culture. The tendency for outgrowing fibres to form large parallel cable-like structures was noted. Migration of granule cells along these fibre bundles was observed by time-lapse cinematography and electron microscopy. Such migration was not seen in similar cultures of embryonic midbrain or cerebral cortex (Hatten and Sidman, 1978).

Succinyl concanavalin A, a dimeric form of a plant lectin, inhibited
cable formation in embryonic cerebellar cultures, this inhibition was blocked by pre-incubation with methyl α-D mannoside suggesting a role for cell-surface carbohydrate-containing macromolecules in the formation of these region-specific patterns (Hatten and Sidman, 1978).

Other specific brain areas in culture

Although the greatest volume of work has been done on the cerebellum, other specific regions of the brain have been studied in dissociated cell culture. These include hippocampus (Banker and Cowan, 1977), cerebral cortex (Dichter, 1978), and hypothalamus.

Hypothalamus from perinatal rats has been cultured successfully for several weeks (Wilkinson et al, 1974). This work was most remarkable for the apparent culture of hypothalamic neurons from 4-5 weeks old rats, considerably more mature than in any other report in the literature. Recoveries were very low, and a very rich medium was used. Embryonic mouse hypothalamus cells have been cultured (Benda et al, 1975) for several weeks, synapse formation was shown by electron microscopy. Knigge et al (1977) have cultured basal hypothalamus from 10-12 day old rats and found neuron survival up to 3 months. One of the most interesting features of hypothalamic cultures is the probable presence of neurons containing specific hormones and releasing factors. Knigge et al were able to stain 20% of neurons in their cultures with an antibody to luteinising hormone releasing hormone (LHRH). A small proportion of the other neurons showed catecholamine fluorescence by the glyoxalate method. These authors stated their aim as study of the factors regulating hormone secretion from hypothalamic neurons. Long-term cultures such as they have developed seem an excellent system for this purpose.
Cultures of rat embryonic cerebral cortex cells have been studied by electrophysiological methods (Dichter, 1978). Neurons of "pyramidal" and "stellate" appearance differentiated over time in culture, and complex post-synaptic potentials were detected. No direct effect of a mitotic inhibitor on electrical activity was found and it was suggested that the results of Godfrey et al (1975) might be due to overgrowth of uninhibited cultures by non-neuronal elements. The detection of recurrent excitatory and inhibitory synapses was discussed in terms of the fundamental role of such systems in the intact CNS. It is possible that new information on the specificity of these connections and on the basis of the neuronal membrane potential can come from studies on this culture system.

Hippocampal cells have been grown in culture in a study which has several unusual aspects (Banker and Cowan, 1977). Cells were cultured at very low densities on polylysine substrates. Neurons were seen to attach and form processes in isolation from any other cells, although their survival beyond five days (to two weeks) was dependent on co-culturing with explants of hippocampus, presumably providing essential factors in the medium which are provided by other cells in higher density cultures or by the addition of NGF in sympathetic ganglion cultures. No problem with overgrowth by non-neuronal cells was found under these conditions; very little gliogenesis is taking place in the rat hippocampus at this age. Hippocampal neurons were found to grow more slowly and have much smaller growth cones than dorsal root ganglion cells, and these
differences were adduced to be at least in part due to inherent differences in programming. Thymidine incorporation revealed that there was preferential survival in culture of early postmitotic cells, those which completed their last division 12-48 hours before dissociation of the tissue. The authors suggest there may be comparable critical periods in the life of other neurons when they are best able to survive the rigours of dissociation and dispersion in culture.

The very low density of these hippocampal cultures has permitted a detailed analysis of the pattern of process outgrowth (Banker and Cowan, 1979). Dendrites were distinguished from 'axon-like' processes. The former arose from the perikaryon as thick, tapering processes, branched at acute (Y-shaped) angles to give daughter processes of reduced diameter, and like dendrites in vivo were capable of transporting and accumulating $^{3}$H-uridine-labelled RNA over 24 hour incubations (cf Kreutzberg and Schubert, 1975). The dendritic organisation was found to substantially resemble that of hippocampal pyramidal cells in vivo, despite the lack of all direct cellular interactions, and this was concluded to indicate an inherent programming of this basic pattern. However elaboration of higher-order dendritic branching was significantly reduced in cell culture. 'Axon-like' processes were fine and uniform in diameter, branches tended to be at right angles to the parent and of similar diameter, and no labelling of RNA occurred. In contrast to dendrites, the axon-like processes extended for great distances in culture (one cell was found to have a minimum total axonal length of 18 mm.)
but differed from cells in vivo in their general organisation. Although sometimes found to arise from a single basal point on the perikaryon (as in vivo), they also grew from inner dendritic segments, occasionally from two or three different points. Processes from cells in young cultures could not be so classified and the authors suggest that their low-density cultures may provide a particularly favourable situation for analysing differentiation into axons and dendrites, a crucial event in neurogenesis.

The culture of neurons from embryonic chick brain in the virtual absence (0.1%) of glial cells has recently been briefly reported (Pettman et al., 1979). Mitotic inhibitor was not used; it appears that, in contrast to the rat, polylysine coated surfaces suppress division of the few glia present in this immature chick tissue.

Brain glial cultures

There is one brain cell type which, due to its ability to divide in culture, has been available in fairly pure primary culture for several years and which has yielded much biochemical data of considerable importance. Booher and Sensenbrenner reported in 1972 that when dissociated brain cells from chick embryos older than 14 days were plated, neuronal cells degenerated during the first week and after 2 weeks in culture only 'mesenchymal' cells remained, which have been subsequently described as a nearly pure glial population (e.g. Sensenbrenner, 1977). Culture of dissociated newborn rat or mouse brain cells was similarly found to yield a monolayer of glial-like cells, the neuroblasts dying off in the first two days in vitro (Booher and Sensenbrenner, 1972). The cultures developed high levels of glial fibrillary acidic protein (GFAP), a marker for astrocytes (Antanitus et al., 1975). Although the cells were generally of a flattened 'epithelioid' morphology when surviving and multiplying
in monolayer culture, they could be induced to assume a process-bearing star-like aspect within a few hours in the presence of dibutyryl cyclic AMP (Shapiro, 1973, Lim et al, 1973). Lim and co-workers have made an extensive study of an apparently similar morphological "differentiation" which occurs in cultured glia when exposed to a protein factor found in crude extracts of adult brain, and has been partially purified, and designated Glia Maturation Factor (GMF) (Lim et al, 1977a). A two-stage response is seen; the first detectable chemical response to addition of GMF is an increase in intracellular cyclic GMP after a few hours, this is succeeded by rounding up of cell bodies, the formation of processes, and a surge of cell division. At 3 days there is a decrease in cell division, and in the motility of cell processes, a three-fold increase in levels of intracellular cyclic AMP, and ten-fold increase in S-100 protein (Lim et al, 1977b). The effects of GMF thus have a different time-course to those of dibutyryl cyclic AMP, which subside within 24 hours. It has been suggested that GMF may be a surface-bound intercellular message present on glioblasts which is of significance in the control of their proliferation and differentiation in vivo (Lim et al, 1977a).

Many biochemical studies have been made on cultured glia (for review see Hertz, 1977); they have particularly contributed to available data on glial amino acid uptake and metabolism which may be important in the control of intercellular levels of putative amino acid neurotransmitters such as GABA and glutamate (reviewed by Schousboe, 1977; Bamberger et al, 1977; Hertz, 1977). Attention has been focussed on this area by the hypothesis that GABA (Van den Berg and Garfinkel, 1971) or glutamate (Benjamin and Quastel, 1975) is
released as neurotransmitter by neurons, taken up by glia, metabolised to glutamine which is not physiologically active, then released for re-uptake by neurons, and re-conversion to transmitter. High affinity uptake of GABA and glutamate has been demonstrated in cultured astrocytes (Schousboe et al, 1977; Hertz et al, 1978) as it had in brain slices (Balcar and Johnston, 1972) and in glioma cell lines (Schrier and Thompson, 1974), however in the latter case the $V_{\text{max}}$ was several-fold lower, while primary astrocytes were found to be comparable to brain slices (Schousboe, 1977).

The usefulness of kinetic parameters calculated in a single system is demonstrated by Hertz (1977) who compared rates of uptake of GABA, glutamate and glutamine with activities in astrocyte cultures of the enzymes which interconvert these amino acids and intermediates of the TCA cycle. From these combined data he was able to calculate the likely contribution of astrocytes to the metabolism of GABA and glutamate in vivo. He concluded that the uptake and conversion of glutamate are high enough to account for any possible flux connecting neuronal and glial metabolism, as proposed by Benjamin and Quastel (1975). However the activity of GABA-transaminase and the rate of GABA uptake are only high enough to account for a part of the degradation of GABA via the 'GABA shunt' observed in vivo (Balazs, et al, 1970) and therefore does not support the concept (Beart et al, 1974) that the total degradation of GABA is located in glial cells. Presumably the remainder is located in neurons, but there is of course no equivalent homogeneous culture in which to determine levels of amino acid metabolism.
More recent results on the uptake and metabolism of glutamine (Schousboe et al., 1979) are in general agreement with the hypothesis of a neuronal-glial glutamate-glutamine cycle. Substantial release of glutamine, and no high-affinity uptake by astroglia was found, suggesting that high-affinity glutamine uptake seen in brain slices (Balcar and Johnston, 1975) is neuronally located. However, given the suggested high in vivo extracellular concentration of glutamine (e.g. 500 mM; Gjessing et al., 1972), the low affinity concentrative net uptake found in astroglia will account for a significant minor component of total glutamine uptake. In accordance with this is the surprising finding of a considerable glutaminase level in astrocytes which clearly does not fit in with the simple cycle hypothesis. The authors conclude that a part of glutamine breakdown must occur in astrocytes, although the possibility that glutaminase may be induced in cultured astrocytes by high medium levels of glutamine is also discussed.

CONTINUOUS CELL LINES

In 1940 a mouse at the Jackson Laboratories was found to have a paravertebral tumour which has been carried since that time by serial transplantation, and, more recently in cell culture. This is the origin of the "neurobiologists Hela cell", the mouse C-1300 neuroblastoma. A very large amount of work has been done on these cells, probably more than on all primary culture systems combined. No review will be attempted here. Work on clonal cell lines has formed the basis of several books (e.g. Sato, 1973) and a recent review has appeared (Patrick et al., 1978).
In the intact tumour, neuroblastoma cells lack processes but when first established in culture, rich networks of long processes were seen to develop (Augusti-Tocco and Sato, 1969; Schubert et al., 1969). By the use of inhibitors, protein synthesis was found to be not necessary for process extension, but the colchicine-sensitive assembly of microtubule units into extended microtubules was required (Seeds et al., 1970). Clones of C1300 neuroblastoma have been isolated that synthesise different neurotransmitters (Amano et al., 1972; Breakefield, 1976), have different pharmacological properties (Gilman and Nirenberg, 1971), and express different morphologies. There are also aberrations in chromosome number, all of these variations are probably due to unselected mutations which have arisen during the large number of in vivo tumour generations. Selection techniques have been applied to obtain clones with desired characteristics (Breakefield and Nirenberg, 1974). In order to enlarge further the spectrum of cell lines with neuronal properties C1300 cells have been fused with other cell lines, and hybrid cells selected (Minna et al., 1972). Hybrid neuroblastoma/glioma lines have been successfully used to study the opiate receptor (Klee and Nirenberg, 1974) and cell-cell interactions, including synapse formation with cultured muscle (Nelson et al., 1976) a capacity not displayed by any C-1300 clone.

In the last few years, a technique has become available for induction of brain tumours by transplacental administration of nitrosourea. Schubert and co-workers have used this method to produce a large collection of clones with few chromosomal aberrations, some of which express neuronal properties, although most are of glial
origin (Schubert et al., 1974; Patrick et al., 1978). Immature cells growing in primary culture may also be transformed chemically or virally. A nerve cell line synthesising neurophysin has been selected from the hypothalamus in this way (de Vitry et al., 1974). This method has considerable potential as a means of transforming cells of known origin and properties in the hope that some of these differentiated properties will be retained in the resulting cell line. It has also proven possible to obtain cell lines from primary cultures of cerebellum and other brain areas without the use of external transforming agents by long-term culture and careful selective sub-culturing (Bulloch et al., 1977). Cells of 'neuronal' and 'glial' character were obtained by these workers, however they are not normal cells, most are tumourigenic when injected into animals, this is merely a different, possibly superior, way of selecting transformed cells of neuronal origin.

Perhaps the clonal cell line studied in most detail besides the C-1300 clones is the sympathetic-like clone PC12 (Greene and Tischler, 1976) which derives from a rat pheochromocytoma. These cells synthesise catecholamines, store them, and most interestingly, respond to nerve growth factor with extension of neurites and cessation of division. The response to NGF has been found to involve no changes in the pattern of protein synthesis detected by a two-dimensional gel system (Patrick et al., 1978). PC12 will also synthesise acetylcholine and synapse with skeletal muscle (Schubert et al., 1977). The clone is thus phenotypically quite similar to primary cultures of sympathetic ganglion cells although it derives from a tumour of the adrenal medulla.
Clonal cell lines are in theory an ideal system for the biochemist to study neuronal differentiation. They present an attractive prospect of dividing populations of identical cells which can be grown in any quantity and which will, in response to set cues, display many aspects of neuronal differentiation. The reality has been far more complex and in some respects disappointing. As with all culture systems, clonal cell lines have given useful information when used to answer suitable questions. However expectations of a few years ago that the mouse neuroblastoma would prove an ideal in vitro model of neuronal differentiation have certainly not been fulfilled.

CULTURE MODELS

It is possible to generalise from the last point to the broad question of the usefulness of nervous system culture models. The essence of a useful model is the drawing of a parallel between what happens at some level of organisation in vivo and what is happening at a similar level of organisation in vitro, but in conditions where it is more easy to study. This concept of the level of organisation is important, and clearly the necessary level must be reproduced in the culture system used. No-one would suggest that clonal cell lines are a good system for the study of factors controlling cortical layer formation. However more subtle errors in drawing this parallel may have led culture work up some blind alleys.

The parallel for explant culture is at the level of tissue organisation. The working assumption is that the culture is reproducing
many of the properties, including spatial arrangement, of the original tissue, but in a more controllable environment. The disadvantage of course is that for this very reason, the number of methods available for study of the system remains limited - particularly for the biochemist. For primary cell culture the parallel is between cells. As has already been argued, the behaviour of cells in culture can, or at least should, be related back to the behaviour of the same cells in vivo. Complexities of tissue organisation are not reproduced and cannot be studied, but many aspects of the cell's own development are available for investigation. This level of analysis is also often applied to clonal cell lines in culture, mistakenly in the view of this author. Hence the efforts to determine which clones are "neuronal" and which "glial", often with very confusing results. By their nature, cell lines have modified control of their differentiation, a genetic or epigenetic change has occurred which allows them to proliferate in culture. In addition, during the many generations in culture, other changes occur, including such gross alterations as the loss of chromosomes. Many clones synthesise two or more different transmitters, such as Schubert's B65 which makes acetylcholine, GABA and dopamine (Schubert et al, 1974). No such cell is likely to exist in vivo, however it may be a good model for the study of the enzymes associated with those transmitter systems.

Thus clonal cell lines can be of great importance at the level of molecules or of macromolecular systems and this is the parallel which should be drawn. They may reproduce, in a highly tractable form, specific neurochemical systems. For instance the PC12 line responds to NGF and may be a system for study of the molecular basis
of that response. The cell seems to behave in this respect like a sympathetic ganglion cell. The fact that it derives from adrenal medullary cells may be of little importance as long as this analytical approach is adhered to.

The emphasis of my own work, and this review has been on cell cultures from the brain. I have suggested that they offer an attractive compromise between the, until now, more widely used, explant cultures and clonal cell lines; the former presenting problems of tissue complexity and very small quantities, the latter being impossible to relate back to an in vivo counterpart. Primary brain cell cultures are not going to prove the in vitro system in which to answer every biochemical question on brain development any more than did the neuroblastoma. However they do offer one very useful model, a model which has been under-exploited up to now. I have tried to show that some problems could be resolved if the system was properly and rigorously defined with respect to cell composition and preferably a range of simplified, or even purified, cell cultures was available. The remainder of this thesis will be devoted to a description of work which makes some steps, albeit incomplete ones, toward this goal.

When R.G. Harrison initiated the study of nervous tissue in culture at the beginning of this century (Harrison, 1910, see frontispiece), he did so to answer the leading neurobiological controversy of the day. Cajal believed that all nerve fibres arose from single cells, while Held and others maintained that a syncytium of interconnecting fibres was formed by neurons in interaction with the
surrounding matrix. Harrison's experiments showing the outgrowth of fibres from nerve cell bodies largely resolved this impasse in favour of the former view. "The experiments show that neuroblasts are competent to form primitive nerve fibres within a foreign unorganised medium simply by the amoeboid outgrowth of their protoplasm. By eliminating from the periphery all formed structures which have heretofore been supposed to transform themselves into nerve fibers and leaving only the neuroblasts in the field, it is demonstrated that the latter are the sole elements essential to the formation of nerves. The concepts of both Hensen and Held are rendered untenable." (Harrison, 1910, page 842). There has been no better demonstration of the power of the right culture model to answer questions which cannot be resolved \textit{in vivo}. 
This chapter describes the first stages in the preparation of defined cerebellar cell cultures. Firstly, optimal conditions were established for the survival and differentiation of the total cell suspension, prepared from postnatal rats as in Chapter 2. Secondly, the morphological differences between cells, and their progress with time in culture were monitored at the light microscopic level; some attributions of cell type could be made. Thirdly, the separated fractions prepared by unit gravity sedimentation (Chapter 3) were cultured, and this provided further evidence on the identity of the fractions established by Coulter analysis. The method was extended to the culture of other brain areas (olfactory bulb, hippocampus and cerebral cortex) from the rat, and cerebellum from postnatal mouse. Almost all of the evidence presented here consists of phase contrast light micrographs, these are adequate for monitoring the general condition of the cultures but can only provide limited information on the cell types present in culture. Further evidence on cell identification, using more specific markers for cell type will be presented in Chapter 6.
METHODS

All operations were carried out in a laminar flow sterile hood (Microflow). Culture dishes, pipettes, and syringes were disposable plasticware (Falcon, Sterilin). Glass and metal items were sterilised by dry heat at 160°C, rubber and plastic items by rinsing with 70% ethanol and allowing to dry. All solutions and media were finally dispensed by a syringe through a Millex 0.22μm disposable filter unit (Millipore (U.K.) Ltd.).

The starting material for primary cultures was the total suspension of dissociated cerebellar cells (TCS) provided by the method described in Chapter 2. The final pellet of cells was resuspended by gentle pipetting in a few drops of culture medium and plated at a normal density of 0.2 x 10^6 cells per cm^2. Cultures were also prepared from the enriched fractions of cells obtained by the separation method described in Chapter 3. In this case, following Coulter analysis and centrifugation of the separated fractions, the cell pellets were resuspended in the same manner, pooled where appropriate and plated at 0.2 x 10^6 cells per cm^2 on the basis of the cell counts obtained during the analysis. Occasional Coulter counting of the combined fractions after resuspension showed that losses during centrifugation were negligible. Dissociated cultures were also prepared from rat olfactory bulb, hippocampus, and cerebral cortex, and from mouse cerebellum. Cell preparation from these tissues and, where performed, separation at unit-gravity, were by the same methods.
Culture dishes used were 6cm and 3.5cm tissue-culture treated polystyrene petri dishes by Falcon or Lux (via Eurolab). Cultures were also grown in Falcon 24-well Multi-well dishes, each well being 17.5 mm diameter, and sometimes on circular glass cover slips (Chance, 13mm diameter) placed in dishes or Multi-wells. Dishes were coated with Poly-L-lysine (>100,000 molecular weight, Sigma) by rinsing them once with a 8 µg/ml solution in water and allowing to dry. Cover-slips after sterilisation were dipped in poly-lysine solution and allowed to dry.

The standard medium conditions were based on Eagle's Minimal Essential Medium with Earle's salts and without L-glutamine (Gibco Bio-Cult Ltd., Scotland). The medium was supplemented with 2.0 mM L-glutamine (Sigma); 10% foetal calf serum (Bio-Cult); 2.5% of 50% chick embryo extract (Flow Laboratories Ltd., Scotland, centrifuged at 50,000 g for 2 hours); 0.6% glucose; 18.5 mM KCl (giving final concentration of 24 mM); and 100 µg/ml of gentamycin (Flow Labs.). After 24 hours in culture the medium was supplemented by the addition of 8 x 10^-5 M fluorodeoxyuridine (FUdR, Sigma).

Other versions of the culture medium are discussed in this chapter. They include the substitution of 10% horse serum (Bio-Cult) for foetal calf serum; the use of 100U/ml penicillin and 100 µg/ml streptomycin instead of gentamycin; the use of Dulbecco's MEM or Ham 's F12 or Eagle's MEM based on HEPES buffer (all from Bio-Cult) instead of MEM with Earle's salts.
Culture medium was freshly made up before use; sterile MEM was kept at 4°C, the gentamycin at room temperature, all other additions were kept as concentrated solutions in frozen aliquots.

Cultures were incubated in a Lab-line CO₂ incubator at 35.5°C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every 3-4 days. The progress of the cultures was observed using a Zeiss Standard inverted microscope with phase contrast optics, and photographs were taken on the same microscope using a Pentax ES camera and Kodak Panatomic X film. Time lapse cinematography of cultures was performed on the same microscope using a water-heated stage built by the Open University workshop to the author's own design. The heated block held a 6 cm Falcon petri dish with a Cooper recessed lid. The dished central area of this lid dips into the medium preventing misting of the lid, which adversely affects the phase contrast image. The lid was sealed between medium changes, and HEPES buffered Eagles MEM was used, thus eliminating the need for a CO₂ atmosphere. The camera was a Vinten 16mm cine camera and control box, using Kodachrome 16 mm Professional film and filming at one frame every one or two minutes.

Incorporation of labelled thymidine was studied in cultures grown on glass cover slips in medium without FUdR. 1μM ³H-Thymidine (5 Ci/mol, Amersham) was added to the culture medium on the first, second, third or fourth day in culture. After 24 hrs, 100 μM 'cold' thymidine was added as a 'chase' to prevent further incorporation. All the cultures were rinsed and fixed on the fifth day (48 hrs in vitro) using 2.5%
glutaraldehyde in Dulbecco's phosphate buffered saline. The cover slips were removed and mounted on microscope slides with the cells on the upper side. The slides were dipped in Ilford L-4 photographic emulsion diluted 1 in 2 with water, drained, and then allowed to expose for 2 weeks at -20 °C. The autoradiograms were developed with Kodak Phen-X and counterstained with haematoxylin and eosin.

Plating Efficiency was determined by comparing the amount of DNA in the cell suspension as inoculated with the amount adhering to three 6 cm. plastic dishes after 24 hrs. Cultures were rinsed with fresh medium, then treated with 0.1% trypsin for 10 mins. and scraped. DNA estimation was by the method of Zamenhof et al (1964).

RESULTS

Modifications of Culture Conditions

The first cultures of the total cell suspension were grown on plastic dishes in a conventional medium of Eagle's MEM with 10% foetal calf serum, 0.6% glucose, penicillin and streptomycin. From the beginning cultures grown in such a medium contained cells of several different morphologies including apparent neurons. The final optimum conditions described in the previous section were arrived at after variation of most aspects of the culture conditions resulting in several important improvements.

Different media were tried and found to give poorer results
than Eagle's MEM although preferred by other investigators for primary
culture of the nervous system. These included Dulbecco's modification
of Eagle's MEM (which has doubled levels of amino acids and co-factors
and a very high level of bicarbonate) and Ham's F12 medium which
includes a wider range of cofactors and trace elements (e.g. Zn, Fe)
and differing levels of amino acids. The substitution of horse serum
for foetal serum resulted in a lower level of initial survival
(Fig. 5.1) although did not affect the long-term survival of the cells.
In fact there was some evidence for a preferential survival of cells
in horse serum after 2 weeks in culture (Fig. 5.2). This has been
reported by other workers (Godfrey et al., 1975; Messer, 1977a) but
horse serum was not found to be consistently necessary for long-term
survival in cerebellar cultures (Fig. 5.2g,h). It is likely that
some batches of foetal calf serum are in some way deficient or
toxic for more mature cultures, horse serum not being so prone to
batch difference. Calf serum was found to be generally inferior
to foetal calf serum for all purposes. Initial survival was also
promoted by the addition of 2.5% of the soluble fraction of a 50%
chick embryo extract (Fig. 5.3).

However, the most important element in the initial survival of
neurons in culture is the nature of the culture surface. If cells
were plated onto a surface, such as untreated glass, to which they
were slow to adhere, then a degree of initial aggregation of cells
before attachment occurred. The best cultures always resulted
from conditions where all cells attached singly to the culture
surface in the first few minutes. Conversely, the presence of
floating aggregates of cells in a young culture was a sure sign of
a toxic influence or some non-optimum condition. Cerebellar cells
were viable on glass surfaces and gave better results on tissue culture
Figure 5.1: Total cell suspension from 7-day postnatal rat cerebellum, cultured in vitro for 4 days (R7 - TCS, 4 DIV).
A: 10% Horse serum (HS) from plating instead of foetal calf serum (FCS); B: FCS control. There is an increased level of cell death in cultured plated in HS, however those neurons which survive this initial period then seem at least equally viable in subsequent culture. (See Figure 5.2). Scale bar 50μm.

Figure 5.2 (opposite): R7 - TCS; a, c, e: HS culture, 16 DIV, 19 DIV, 22 DIV respectively. b, d, f parallel FCS culture, 16 DIV, 19 DIV, 22 DIV respectively. It is apparent that this batch of FCS contains some deficiency or toxic influence which causes the onset of neuronal death around 19 DIV. However some batches of FCS can support long term culture as shown in a different R8 - TCS preparation. g: 33 DIV, HS culture. h: parallel FCS culture. Scale bar 50μm.
Figure 5.3: Effect of chick embryo extract (CEE), R7 - TCS, 7 DIV. a: Culture without CEE b: parallel control culture. Chick embryo extract was found to give an initial improvement in cell survival, but as shown in a, subsequent survival is not qualitatively dependent on the presence of CEE. Scale bar 50μm.
treated polystyrene surfaces. However a notable improvement on
either was provided by coating the surface (either glass or plastic)
with poly-L-lysine. (Fig. 5.4). Cells then adhered swiftly to the surface and
only formed aggregates by migration after several days. A polymer
of another basic amino acid, poly-L-ornithine was found to give
similar results to polylysine in this system. Plating efficiencies,
obtained by comparing the amount of DNA in cells adhering to the dish
after 24 hrs. with the amount in the cells at plating, are doubled
by poly-L-lysine coating. However, this rise (from 40% to 90% in
the TCS, see Table 1) may be more apparent than real: it is likely
that non-viable cells adhere to the poly-lysine surface and then lift
off after 2-3 days. The effects of polylysine are short-term;
there is little difference with or without polylysine beyond one week
in culture.

Survival of neurons beyond the first few days in culture depends
on prevention of overgrowth by dividing non-neuronal cells. Addition
of the mitotic inhibitor fluorodeoxyuridine (FUDR) after 24 hours
in culture was found to be optimum in this system (Fig.5.5 ). Cultures
without FUDR were not badly overgrown by 3 or 4 days in vitro, but
there seemed to be a delay in FUDR effectiveness, and addition at this
time was not sufficient. Use of FUDR was first suggested by Godfrey
et al (1975). Overgrowth by dividing flattened non-neuronal cells leads
to a loss of recognisable neurons by one week in culture (Fig.5.5).
Subsequent studies using markers for cell type (see following chapter)
have shown that the great majority of the flattened non-neuronal
cells are astrocytes.
Figure 5.4: Effect of poly-L-lysine coating, R9-TCS, 2 DIV. a: Cells plated on untreated glass surface, most neurons are already aggregated. b: Parallel culture, cells plated on poly-lysine coated glass, note that a far greater proportion of neurons have attached singly to the surface. Scale bar 50 μm.
Figure 5.5: Effects of FUdR. a: R7 - TCS, 4 DIV, with $8 \times 10^{-5}$ M FUdR added after 24 hrs in vitro. b: A parallel culture without FUdR, note the increased background of flat cells, however neurons are still surviving well. c: same culture as a, 6 DIV. d: same as b, 6 DIV, with FUdR since 4 DIV, however serious overgrowth is not prevented by this later addition, neurons are obscured by background cells and will die off by 9 DIV. e,f : R8 - TCS, 12 DIV, growth without FUdR results in a culture of flattened cells (predominantly astrocytes, see Figure 6.10). Scale bar 50μm.
Figure 5.6: Effects of elevated K\(^+\) level. R7 – TCS. a, c, e: 4 DIV, 7 DIV, 11 DIV respectively. Culture with medium containing conventional 5.3mM K\(^+\), neurons die off in second week in vitro. b, d, f: 4 DIV, 7 DIV, 11 DIV respectively. Parallel control culture containing 24mM K\(^+\). Scale bar 50\(\mu\)m.
For long-term survival the most important modification of the culture conditions was elevation of the potassium ion concentration from 5.3 mM to 24 mM. This gave a slight improvement in the apparent condition of the neurons during the first week in culture but after 7 days the difference became dramatic. Neurons disappeared from all low K⁺ cultures within two weeks, but survived to 5-6 weeks with high K⁺ (Fig. 5.6). The final modification was to use gentamycin in place of penicillin and streptomycin which have been shown to be unstable at 37 °C, losing 80% of their activity within three days. Gentamycin prevented the occasional incidence of gross bacterial contamination, but more importantly produced a general improvement in consistency of culture quality, possibly by prevention of low-level contamination.

The growth of cerebellar cells in culture

Figure 5.7a shows the typical appearance of a freshly plated culture of cerebellar total cell suspension (TCS) from 6 to 8 day-old rat. In about 30 minutes the cells attached singly to the polylysine substrate, and began to flatten out. Processes were extended within a few hours and after 24 hours there were clearly many cells of differing morphologies (Fig. 5.7b ). However after three days the differences between cells were more apparent (Fig. 5.8). The most numerous population of cells had small (<10 μm) rounded cell bodies which appeared bright in the phase contrast microscope. They extended bipolar, or multipolar, processes which formed a complex network in the
culture. By their large numbers and their morphology, these cells may be tentatively identified as granule neurons. There were also smaller numbers of large, flattened non-neuronal cells of the appearance generally attributed to glial or other non-neuronal cells.

Table 5.1 Plating Efficiency of cerebellar cells, i.e. amount of DNA adhering to plastic dish after 24 hrs. compared with amount in inoculum.

<table>
<thead>
<tr>
<th>Cell Suspension</th>
<th>Plating Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCS</td>
<td>42%</td>
</tr>
<tr>
<td>Fraction B</td>
<td>39%</td>
</tr>
<tr>
<td>Fraction C</td>
<td>73%</td>
</tr>
</tbody>
</table>

These figures are for untreated polystyrene dishes, poly-L-lysine coated dishes gave a Plating Efficiency of 80-90% in all these cases, but see text for discussion.

In addition there was a proportion of cells with a large (~20 μm) well-defined cell body and multiple processes, which could be of astrocytic or neuronal character.

By the sixth day in culture (Fig. 5.9) there were signs of the small neuronal cell bodies coming together into aggregates. The complexity of the network had further increased, and processes were beginning to associate into bundles. The main feature of the cultures during their second week is the migration of the putative granule cell bodies into aggregates, some as large as 100 cells, which are connected by bundles of neurites up to 10 μm in diameter.
Figure 5.7: Growth of cerebellar TCS in vitro. a: R8-TCS, at time of plating. b: R7-TCS, 1 DIV. Note that many small rounded cells are already extending long processes, while other cells are flattening, some also with processes. Scale bar 50µm.
Figure 5.8 (montage): Growth of cerebellar TCS, R7-TCS, 4 DIV. A variety of cell morphology is apparent at this age, note the many small rounded phase-bright cells which are already showing some tendency to group together and from which an extensive network of processes is already emerging. There are also many flattened background cells, often difficult to discern, some are marked F near their nuclear region. Other larger cells bear processes, some are marked P. Scale bar 50µm.
Figure 5.9: Growth of cerebellar TCS. a: R7-TCS, 6 DIV. Neuronal aggregates are clearly forming, and the processes are beginning to form bundles. Some large flattened background cells can be clearly seen. b: R7 - TCS, 8 DIV. A particularly striking example of the size of some neuronal aggregates and process bundles, the large fascicle shown must contain many hundreds of processes. Scale bar 50μm.
Figure 5.10: Growth of cerebellar TCS. a: R8 - TCS, 22 DIV. The interconnecting network of fibres can be highly complex. b: R8 - TCS, 33 DIV. The neuronal cells and processes can remain stable until the sixth week in culture. However degeneration takes place after this age. Scale bar 50µm.
Figure 5.11 (montage): R8 - TCS, 28 DIV. This montage of a large area of culture shows that the great majority of small putative granule neurons are in aggregates, with their processes fasciculated. Scale bar 100µm.
and containing many hundreds of individual processes (Figs. 5.9b; 5.10a)
This general morphology then remained from the second to the sixth
week (Fig. 5.10b) when neuronal degeneration began to occur. Figure 5.11
shows a montage of a mature culture which illustrates the final
result of the cell migration in culture, over 95% of the putative
granule neurons are in aggregates with their processes in large
parallel bundles.

These changes in culture were monitored and described on the
basis of daily observation by phase contrast microscopy and photography.
Time-lapse cinematography revealed further details. It immediately
became clear that the overall changes seen from day to day were only
the sum of incessant cell movement. Cells in culture were constantly
changing in shape and position. This was particularly true during
the first few days in culture. But even the older more 'stable'
cultures were constantly changing in the detailed arrangements of the
cells and cell aggregates. The time-lapse work also showed some
details of cell movement. The flattened cells moved by the
advancement of a leading lamella podia showing the classic ruffling
membrane. However single putative granule neuron cell bodies could
often be seen moving along their own bipolar processes. This kind
of movement had not previously been reported and is particularly
interesting in the context of the suggestion that the migration of
the granule cell in vivo is actually a translocation of the cell body
down a leading process (Altman, 1975).

More recently Trenkner and Sidman (1977) have reported the movement
of putative cerebellar granule neuron cell bodies along bundles of processes. This is almost certainly an example of the same phenomenon which is seen here more clearly in single cells.

Cell division in culture

Figure 5.5 has already illustrated the overgrowth by non-neuronal cells seen in cultures where division is permitted (i.e. FUdR is omitted). The nature of the cells capable of division in culture was further investigated by an autoradiographic study of $^3$H-Thymidine incorporation. The protocol involved a 24 hour pulse of labelled thymidine followed by a cold 'chase'. Figure 5.12 shows the unambiguous result. Label was accumulated over the nuclei of the flattened non-neuronal cells and never over the neuronal cell bodies. This was true even for incorporation during the first 24 hours in culture and even for cultures of the 'C' peak fraction which is enriched in dividing granule neuroblasts.

Culture of separated cell fractions

The results of culturing each peak fraction are described below, the cell compositions assigned to each fraction in the previous chapter are repeated here.

Fraction E: Diameter >14.5μm, Purkinje cells. More than half of the large cells which dominate this fraction attached to a poly-lysine coated substrate. However the great majority of cells subsequently
Figure 5.12 $[^3\text{H}]$Thymidine incorporation, R8 - TCS, 4 DIV. Autoradiographic grains are present over the nuclei of flattened non-neuronal cells, but never over the nuclei of granule neurons. 24 hrs. incorporation of $[^3\text{H}]$Thymidine from 2 DIV to 3 DIV followed by 100μM unlabelled thymidine at 3 DIV. Counter stained with haematoxylin and eosin. Scale bar 50μm.
degenerated over a period of 2-4 days in vitro without showing signs of process outgrowth (Fig. 5.13). Occasionally, isolated large cells could be seen with some outgrowth of processes but these were a tiny proportion (&lt;&lt;1%) of those plated, and thus very unrepresentative. This lack of survival was not ameliorated by different culture conditions including elevated levels of foetal calf serum and chick embryo extract. Nor was it due to the separation of the Purkinje neurons from other cell types as there was no improvement in survival of E cells plated onto actively growing cultures, or with a proportion of C cells. It is hard to establish definitively whether the E size range cells survive in cultures of the TCS, however degenerating large cells are often seen in TCS cultures. It seems most probable that the E size range are not viable when isolated, rather than being further damaged by the cell sedimentation procedure. The cell separation was effective in that Fraction E cultures contained very few flattened non-neuronal cells, and FUdR treatment was not required.

Fraction D: Diameter 10-14.5 μm, no assigned composition. Cultures of fraction D were of mixed character which was difficult to interpret on the basis of phase contrast morphology. The largest cells in the fraction behaved like Fraction E cells, remaining spherical and degenerating after a few days. There was a proportion of smaller neurons, probably the largest of the granule neurons. There were considerable numbers of non-neuronal cells which flattened out; Fraction D would overgrow without FUdR treatment. There were also larger (~20μm) cells with a defined cell body and multiple processes
Figure 5.13 Growth of E-fraction cells in vitro. 

a: R7 - E, 0 DIV, cells as plated. 
b: R7 - E, 1 DIV, there is very little process outgrowth from E cells after 24 hrs (cf. TCS or C fraction) 
c: R9 - E, 5 DIV, most perikarya are shrunken and degenerating, however occasional cells show some process outgrowth. Scale bar 50μm. 
d, e, f: R7 - E, 4 DIV, examples of E cells which attach and put out processes in culture; these are an unrepresentative minority, for instance, four such cells were found in a well which had been seeded with $3 \times 10^5$ E cells. Scale bars 50μm.
Figure 5.14: Growth of D-fraction cells in vitro. a: R7 - D, 0 DIV, cells as plated, b R8 - D, 5 DIV. Many granule neurons are apparent, but also several intermediate-sized process-bearing cells which may be astrocytes or neurons. Scale bar 50 μm.
Some of these are probably astrocytes, and some may be neurons, probably inhibitory interneurons on the basis of their size. This fraction clearly illustrates the need for better methods of cell identification.

**Fraction C**: Diameter 8-10 μm, dividing external granule neuroblasts and immature granule neurons. This fraction had a very high viability. The plating efficiency was 70% on a plastic surface, compared with 40% for the TCS. It gave cultures of similar morphology to the TCS consisting mainly of small neurons, probably granule neurons, and a proportion of flattened dividing non-neuronal cells (Fig. 5.15).

**Fraction B**: Diameter 6.5-8 μm, immature and pre-migratory granule neurons and possibly dividing external granule neuroblasts in the G-1 phase of the cell cycle. The level of cell death on plating from this fraction was similar to the TCS, perhaps reflecting a proportion of Bp cells (see below). In other respects the B fraction was similar to the C fraction in culture. The most common cell type was the putative granule neuron (Fig. 5.16). There were some flattened non-neuronal cells, though in smaller numbers than in the C fraction, however FUdR was still required to prevent overgrowth by non-neuronal cells. There was a greater degree of aggregation of B cell cultures than of C cell cultures, but the main impression is of the similarity of B and C at least in regard to the most numerous cell type, the granule neuron.
Figure 5.15: Growth of C-fraction cells in vitro, R7-C. a: 0 DIV, cells as plated. b: 1 DIV. c: 4 DIV d: 16 DIV. This fraction follows a similar course to TCS and fraction B. Scale bar 50μm.
Figure 5.16: Growth of B-fraction cells in vitro, R7 - B. a: 0 DIV, cells as plated. b: 1 DIV. c: 4 DIV. d: 16 DIV. There is perhaps greater early aggregation of B cells when compared to C (Fig. 5.15), however compare with Figure 5.17. Scale bar 50μm.
Figure 5.17: Growth of Bp-fraction cells in vitro, R7 - Bp (from the same separation as Figs. 5.15 and 5.16). a: 0 DIV. Note the large numbers of apparently intact cells, and the considerable similarity to B cells (Fig. 5.16a). b: 1 DIV. After 24 hrs. there is a complete contrast with B (Fig. 5.16b). c: 4 DIV. There remain few viable cells in Bp cultures. Scale bar 50μm.
Fraction Bp: 6.5-10 μm, astroglia-like cells. As described in the last chapter, these cells strongly resembled cerebellar astroglia at the ultrastructural level. However the alternative possibility was discussed that they are cells which have suffered some damage, although not sufficient to allow the entry of dye. The culture results tend to support this latter view for Bp cells completely fail to survive in culture. Indeed within 24 hours cells had virtually disappeared leaving only small debris and an insignificant number of growing cells (Fig. 5.17). This is a dramatic result. Bp is usually separated by only four 10 ml fractions (out of 600 ml chamber volume) from B which readily grows in culture.

Fraction A: 5.5-6.5 μm, debris, free nuclei and heavily damaged cells. As would be expected, viable cultures cannot be established from this fraction.

Cultures of other brain areas

A limited number of experiments on the culture of brain areas other than cerebellum were performed, these included olfactory bulb, hippocampus and cerebral cortex. Figure 5.18 shows that viable cultures can be obtained from the olfactory bulb of 5 day-old rats. They include many apparent neurons, most of a size range (<10 μm) comparable with cerebellar granule neurons, and similarly exhibiting some tendency to migrate into aggregates. On the basis of their numbers and size, these cells resemble olfactory bulb granule neurons, possibly including periglomerular and short-axon cells. Unit gravity sedimentation of
Figure 5.18: 5-day rat olfactory bulb cells maintained in vitro.

a: 0 DIV, cells as plated, a greater amount of debris is present compared with cerebellar TCS. b: 4 DIV. Many small neurons, similar to cerebellar granule neurons are present, as are some larger fusiform probable glial cells. c: a separated cell fraction enriched in cells of the B-C size range, note the many small neurons. d: a separated cell fraction enriched in cells of the C-D size range, this is predominantly non-neuronal. Other separated fractions contained few viable cells. Scale bar 50μm.
Figure 5.19: 2-day rat cerebral cortex cells maintained in vitro. 
a: 6 DIV, b: 7 DIV, c, d: 21 DIV. These cultures show little 
evidence of the neuronal aggregation which characterises cerebellar 
and olfactory bulb cultures. Older cultures contain significant 
numbers of large tripolar neurons. Scale bar 50μm.
Figure 5.20: 6-day rat hippocampus cells maintained in vitro. 
a: 7 DIV, b: 10 DIV. The neurons present in these cultures were of 
generally larger size than cerebellar granule neurons, but never 
reached the size of the largest neurons in cerebral cortex cultures. 
Scale bar 50μm.
the olfactory bulb TCS yielded fractions enriched in particular size ranges, however on culture of the separated fractions there seemed to be little enrichment of particular cell types, but only a separation of viable cells from debris and damaged cells. (Fig. 5.18) Cultures of the 2 day-old rat cerebral cortex (Fig. 5.19) were notably different from cerebellum. Many cells of obvious neuronal character were present and these were of generally larger size including a proportion of cells in the range 25-40μm. Even after prolonged (3 weeks) periods in culture, these cells showed no tendency to migrate together into aggregates. A resemblance between some of these larger cells and cortical pyramidal cells must be noted although this author is reluctant to make such an attribution without corroborating evidence. Figure 5.20 shows that hippocampal TCS could also be cultured successfully. The cultures contained many apparent neurons, generally in the size range 15-20μm.

Separation and culture of mouse cerebellar cells

TCS was prepared from the cerebellum of one week-old mouse by the same method and cultured. Cultures took longer to establish, and little outgrowth was observed after 24 hours; however by 4-5 days in vitro there were many apparent granule cells and flattened non-neuronal cells (Fig. 5.21). Indeed the cultures are virtually indistinguishable from those prepared from rat. However during the second week in culture mouse cerebellar cultures were observed to degenerate and lose all neurons; conditions to prolong their life were not determined during the course of the work presented here.
Figure 5.21: Culture of mouse cerebellar TCS and unit-gravity separated fractions. 

a: M5-TCS, 5 DIV, large numbers of aggregated granule neurons and fasciculated processes are apparent. 
b: M5-TCS, 9 DIV. By this age most granule neurons have died off, mainly flattened probable glial cells remain, with some possible larger neurons. 
c: M6-E, 4 DIV, E fraction cells degenerate in culture in a similar manner to rat cells. 
d: M6-D, 8 DIV, most D fraction cells also degenerate, although some possible large neurons survive. 
e: M6-C, 4 DIV, C fraction cells form typical, predominantly granule neuron cultures. 
f: M6-B, 4 DIV. Mouse B fraction cells do not survive well in culture. Scale bar 50μm.
Mouse cerebellum TCS could be separated by sedimentation at unit gravity in the same manner as rat cells, and gave very similar size enrichments as determined by Coulter analysis. Fractions E, D, C, B and A were similarly recognisable, although little Bp peak was observed. When these separated fractions were cultured (Fig. 5.21) fraction E was not found to be viable. Fraction D gave cultures of mixed character, in which a few apparent large (25μm) neurons could be observed. Fraction C gave cultures containing many granule neurons which showed the same tendency to aggregate as did the rat cells. Fraction B however showed a very low viability despite the apparent similarity in appearance of the B and C cells when isolated. No fractions sedimenting more slowly than B contained viable cells.

DISCUSSION

A theme which emerged from the review of culture literature was the heterogeneity of most brain culture systems. In this respect, the cerebellum offers several advantages, it is a well-defined and relatively 'simple' region containing 5 major types of neuron and it has large numbers of cells which mature postnatally. The cell isolation method developed from that of Cohen (as described in Chapter 2) gives a high yield of cells in excellent condition as judged by several biochemical and ultrastructural criteria, and these may be separated into enriched fractions as described in Chapter 3. These cells are therefore promising as a starting material for cultures which
can be defined and compared with their known *in vivo* counterpart.

In several respects, the results described here fulfill this expectation. Cells prepared from one-week old cerebellum attach to the dish and survive with a high plating efficiency. This has the simple advantage that large numbers of cultured cerebellar cells can be prepared from a litter of rats (>10^8). Cerebellar cultures contain cells of several different appearances. The cerebellum is a sufficiently simple source for an analysis to be attempted of the possible *in vivo* counterparts of the cell types seen *in vitro*, however it is complex enough for many ambiguities to remain. Morphology in culture at the light level is a notoriously unreliable criterion for cell identification which has been much over-used in the history of nervous system culture. Work with some markers of cell type described in the following chapter provides further examples of this problem. However one cell type stands out as the most numerous in cultures of TCS and fractions B and C. They are small (<8μm), round, phase-bright, and form long processes usually in a bipolar fashion. By these criteria, and by their sheer numbers, these cells are probably granule neurons.

There is another implication of the high plating efficiency achieved with cells which in themselves are obtained in high yield (up to 50%) from the intact tissue: it may be argued that the cells in culture are to some extent representative of the intact population. About 90% of the neurons in the cerebellum are granule neurons. A high proportion (>80%) of the neurons in culture are
small round neurons resembling granule neurons. As the overall yield from tissue to culture is at least 20%, it is difficult to argue that this majority population in culture are a minority population from the cerebellum which has been selected by the procedure. However there can be no expectation that all cell populations are present in culture in the same proportion as \textit{in situ}. Thus the cultures are more 'representative' of the tissue than many previous systems which have a very low overall yield, but this does not assist in the identification of any cell populations excepting the most numerous.

The kinetic behaviour in culture of the putative granule neurons is also quite characteristic; they migrate actively along their processes and gradually form aggregates while the processes themselves tend to draw together and form parallel bundles. There is an obvious parallel here with the \textit{in vivo} migration at this age of the external granule cells down a leading process to their final position among the densely packed granule cell bodies of the internal granular layer. It is tempting to conclude that the granule migration seen in the 2-dimensional, completely disorganised environment of a culture dish is an inadequate but authentic expression of the specific, directed migration seen \textit{in vivo}. It may also be that the culture phenomenon is unrelated to normal developmental events, but it seems unlikely that an unspecific aggregation behaviour would be restricted to a single cell type. In any case, the possible \textit{in vitro} reproduction of granule cell migration can be borne in mind when interpreting future results.
using this system.

The medium selected as optimal for these cerebellar cultures has one unusual aspect which merits further discussion, the requirement for 24 mM K⁺ level. The importance of high K⁺ for long-term neuronal survival in some cell culture systems was first shown by Scott for the dorsal root ganglion (Scott and Fisher, 1970), and later by Lasher for cerebellar cultures (Lasher and Zagon, 1972). Yet there are many culture systems in which high K⁺ is not required for long-term survival of neurons from the peripheral nervous system (Mains and Patterson, 1973a, Bunge, 1975a) and the CNS (Banker and Cowan, 1977; Dichter, 1978; Trenkner and Sidman, 1977). It is interesting to speculate on the mechanism of this agent. 24mM K⁺ is sufficient to partially, but not fully, depolarise neurons presumably causing a great increase in the spontaneous firing rate. High K⁺ becomes essential about one week in culture, it is in this first week in culture that a several-fold increase in the number of action potential sodium channels per unit protein has been seen by Richard Beale in our laboratory (Beale et al., 1980) using veratridine-stimulated uptake of ²²Na⁺ as an assay (Catterall, 1975). It may also be relevant that the cerebellar granule neuron dies in vivo when its post-synaptic site is missing as in the staggerer mouse mutation which affects the dendritic spines of the Purkinje cell (Sotelo and Changeux, 1974). It is thus possible to speculate that an elevated K⁺ level, by increasing the spontaneous activity of the neurons, in some way substitutes for the lack of normal pre- and post- synaptic elements in culture.

Polylysine was found to improve cell adhesion, as first
demonstrated by Yavin and Yavin (1974). It is likely that it acts via a simple charge-based mechanism; a polymer of another basic amino acid, poly-L-ornithine, has been advocated (e.g. Varon et al, 1979) and was found to give similar results to polylysine in this system.

The lack of mitosis found in the putative granule neurons is perhaps surprising as a large proportion of the external granule neurons exist as dividing neuroblasts in the intact cerebellum at this age. It is clear from the in vivo thymidine incorporation studies described in Chapter 3 that they form a substantial part of the isolated TCS. Yet these cells immediately cease division and begin process outgrowth and differentiation in the culture environment. This may be an effect of trypsinisation or of a lack of some component essential to maintenance of the dividing state. If conditions or factors could be found which promoted neuroblast division in culture, then this aspect of the control of differentiation could be investigated. In addition there would ensue several advantages, including the growth of cultures of virtually pure granule cells in some bulk. This is an exciting prospect, and the cerebellar granule neuroblast is ideal for such studies in that it is available in the dividing state in very large numbers. However there is no clear example in the literature of neuroblasts retaining in culture their ability to divide without undergoing transformation (see Bulloch et al, 1977) and so easy success cannot be expected.

Cultures of the TCS contain many putative granule neurons and so
the excellent survival in culture of the granule-enriched separated cell fractions B and C is a consistent result. The similarity of B and C fractions in culture lends support to the proposition that the two separate populations which were seen in the Coulter analysis (see Chapter 3) are actually different phases in the cell cycle of the same dividing population of cells. However the possibility that B is a postmitotic but still immature (premigratory) population of granule neurons cannot be definitely ruled out. There may also be differences between these fractions in the proportion of less common cell types, in addition to the dividing flat non-neuronal cells, but these must await reliable methods of cell identification before they can be quantified.

Successful culturing of the granule cell fractions indicates that the lengthy sedimentation procedure causes little further damage to the dissociated cell perikarya. Such damage is therefore unlikely to account for the reluctance of separated Purkinje cell perikarya to survive, even in the presence of other cell types, or established cultures. It is yet possible that a vital factor, or change in culture procedure, will allow survival of these large neurons in representative numbers. However it is most probable that the Purkinje cells are too differentiated at one week postnatal age to undergo the cell isolation procedure without suffering irreparable damage. The most fruitful future approach may be to isolate from younger animals, however the cell separation procedure is likely to be less easy to apply as the size differences in younger animals are less pronounced. Finally, culture of Bp, the putative astroglial
fraction, lends strong support to the alternative possibility that Bp is a fraction of cells which have suffered some damage and that it is this damage which accounts for their difference in density from other cells and thus their 'paradoxical' sedimentary behaviour.

The application of this cell isolation and culture procedure to several other tissues, although preliminary, illustrates that the method has wide usefulness. Cells from some of these tissues have been cultured by other laboratories, although there has been no previous report of olfactory bulb cell culture. However the particular applicability of the present method seems to lie in the culture of brain areas at a more mature stage than is possible by other methods. Thus Lasher (e.g. 1974) routinely cultures 2-day rat cerebellum, whereas the present method allows highly viable cultures of 8 day old cerebellum, which contains several-fold more cells, and is particularly 'enriched' in granule cells. Banker and Cowan (1977, 1979) have produced elegant cultures of embryonic rat hippocampus, which are dominated by pyramidal neurons. The present method allows neuron-containing hippocampal cultures to be prepared up to 5 postnatal days which will contain other later-developing hippocampal neurons. It is likely that the reason for this particularly good preservation of viability in cells isolated from more mature tissues lies in the gentle and controlled enzymatic cell isolation method.
**CELL IDENTIFICATION IN CULTURE**

**INTRODUCTION**

The limited identifications of cells in the cultures described in the previous chapter are largely based on morphology in phase contrast microscopy. This has been the main criterion for cell identification throughout the last decades of cell culture. Most of the papers cited in the culture review (Chapter 4) have relied on phase contrast morphology; a few studies have provided further recognition of neurons by electrophysiological measurements (e.g. Nelson and Peacock, 1973; Fischbach and Dichter, 1974), while others have added ultrastructural observations (e.g. Burry and Lasher, 1978a; Trenkner and Sidman, 1977). Some workers have used histospecific stains to distinguish cells, the Holmes silver impregnation is particularly useful for staining neurons and their processes (e.g. Sensenbrenner et al., 1975; Dichter, 1978). Fluorescence of catecholamines induced by the glyoxalate method, and staining for specific hormones were of importance in hypothalamic cultures.
Enzyme histochemistry for acetylcholinesterase and choline acetyltransferase has also been used (Kim and Wenger, 1973). However, these few examples serve as the exception rather than the rule; the great majority of papers on explant and monolayer cultures of central and peripheral nervous system have attributed cell type on the basis of the appearance of the living cells in the phase contrast microscope. This method has proven adequate for the basic distinction between neurons and non-neuronal cells. Non-neuronal cells are flattened, while neurons have a phase-bright rounded cell body with processes emerging, however even this distinction can be tricky. A "differentiated" process-bearing astrocyte can bear a considerable resemblance to a neuron of intermediate size (see below). Oligodendrocytes and Schwann cells also may have a defined cell body bearing processes (Mirsky et al, 1980).

Cultures of the dorsal root ganglion and sympathetic ganglion were reviewed in Chapter 4. The work of Patterson and others on sympathetic ganglion cultures (Patterson et al, 1978) involved an assumption that the neurons in culture could be compared to the neurons of the sympathetic ganglion in situ. This was a reasonable assumption in cultures of peripheral ganglia, which contain a small range of cell types; those cells identified in the culture as large neurons may be a fairly homogeneous population. However, cultures of the central nervous system, no matter how carefully dissected a specific region, must be expected to contain a wider range of cell types. All results obtained on cultures in which the origin of the 'neurons' and other cell types which are recognised in the culture is not known.
present problems of interpretation which have already been discussed at length.

With minor exceptions of limited applicability mentioned above, there has been virtually no method of distinguishing between neuronal types mentioned in the literature. Those few studies in which serious attempts have been made to assign specific cell types to neurons found in CNS cultures have depended on the use of a well-defined area of tissue allied to judgements on the resemblance of the shape of the cell in vitro to that of the putative cell of origin in vivo. This type of analysis is used convincingly by Banker and Cowan (1977, 1979) in their description of hypothalamic pyramidal neurons in dispersed monolayer cultures. Trenkner and Sidman (1977) also use transmission and scanning electron microscopy in their particularly detailed study on cerebellum which depends on the same principles.

There is another method which allows one group of neurons to be distinguished from the remainder on grounds other than size, and that is detection by autoradiography of uptake of \[^3\text{H}\text{GABA}\]. It has been established in vivo that the inhibitory neurotransmitter GABA is accumulated via a high affinity Na\(^+\)-dependent mechanism by glial cells and by some neurons (Schon and Kelly, 1974; Hökfelt and Ljungdahl, 1972). GABA uptake has been concluded (Schon and Iversen, 1974) to be restricted to those neurons which use GABA as their neurotransmitter (GABAergic). The high-affinity uptake systems for GABA in neurons and glia differ in their substrate specificity and can be distinguished using analogues of GABA; thus
β-alanine was found to be specific for glial uptake, while DABA was preferentially accumulated by neurons (Kelly and Dick, 1976). More recently a particularly specific substrate for the neuronal uptake system has been reported, this is cis-1,3-aminocyclohexane carboxylic acid (ACHC) (Bowery et al, 1976).

In the cerebellum, GABA has been found to be accumulated by stellate, basket and Golgi neurons (Kelly and Dick, 1976), which are all inhibitory (Eccles et al, 1967). The excitatory granule neurons, which are of course not GABAergic, did not take up GABA. However Purkinje cells are inhibitory (Eccles et al, 1967) and are believed to be GABAergic (Obata et al, 1967), and yet there seems to be no accumulation of GABA by Purkinje cells in situ, whether administered by in vivo microinjection (Kelly and Dick, 1976) or by incubation of a brain slice (Hökfelt and Ljungdahl, 1972). It has been suggested that Purkinje cell perikarya are surrounded by a glial sheath which prevents access of exogenous GABA (Schon and Iversen, 1974). This explanation of the apparent contradiction of the rule that GABAergic neurons show GABA uptake, is supported by observations that Purkinje cells do indeed accumulate GABA when grown either in explant culture (Sotelo et al, 1972) or in an intraocular transplant (Ljungdahl et al, 1973). Presumably under these conditions the glial sheath is incompletely formed. If it is accepted that a 'naked' Purkinje cell will accumulate GABA, then, of the five cerebellar neuronal types, only one, the granule cell, does not show GABA uptake. This offers a means to distinguish cerebellar neurons growing in culture. Lasher (1974) used [3H]GABA autoradiography to recognize inhibitory neurons in cerebellar cultures. He also showed a low level of uptake into
flattened background cells which he described as "non-neuronal", and designated a few heavily labelled cells with multiple short processes as astrocytes. The smallest rounded neurons, which were present in large numbers did not accumulate $[^3]$H]GABA, and this was argued by Messer (1977) to support their identification as granule cells.

Some results are also presented here on the uptake of $[^3]$H]GABA into cultures of the olfactory bulb. There has been no study of GABA uptake into olfactory bulb in vivo. However there is considerable evidence that the major classes of small inter-neurons in the olfactory bulb, granule cells, periglomerular and short-axon cells, are inhibitory (Shepherd, 1972), and use GABA as their transmitter (Nicoll, 1971; Ribak et al, 1977).

Several immunological makers for cell type have become available recently; although none yet distinguish between different types of neuron, they are of considerable use in identifying the other classes of cell in the culture. The most widely-used marker for glia is antiserum to glial fibrillary acidic protein (anti-GFAP), the major sub-unit of 10nm intermediate glial filaments, which has been shown to be confined to astrocytes in vivo (Bignami et al, 1972), and in culture (Antanitus et al, 1975). Tetanus toxin, recognised by anti-tetanus toxoid, has become accepted as a marker for neurons in culture, it binds to the surface of all neurons in CNS and PNS cultures, and to no other cells (Dimpfel et al, 1977; Mirsky et al, 1978). Thy-1, a surface glycoprotein of unknown function found in
thymus and brain, has been shown on the surface of fibroblasts in CNS cultures (Mirsky and Thompson, 1975); it is also expressed on some neurons (Raff et al, 1979) with a varying time course, and, in the case of cerebellar cultures, depending on the age of origin of the tissue (Currie et al, 1977). Thy-1 has also been observed to develop on astrocytes after about two weeks in vitro (Pruss, 1979), however it remains useful as a marker of fibroblasts, as these are GFA-negative and of flattened shape, quite unlike any neuron. Oligodendrocytes have been recognised in culture by surface binding of an antiserum to galactocerebroside, a glycolipid present in myelin (Raff et al, 1978), and macrophages by their capacity to bind all immunoglobulin (Raff et al, 1979). Schwann cells have been labelled by an antiserum to an undefined surface antigen Ran-1 (Fields et al, 1978; Mirsky et al, 1980). These markers have been used in combination via immunofluorescent double-labelling to define most or all cell types present in cultures of several regions of the nervous system (Raff et al, 1979).

In this chapter will be described the application of a combination of these methods to cultures of the total cell suspension prepared as described above.

METHODS

Cell Culture

Sterile total cell suspension was prepared from postnatal rat cerebellum and olfactory bulb and late embryonic rat cerebellum as described in Chapter 2. Cultures were prepared on polylysine
coated 13μm round glass cover-slips incubated in Falcon Multiwell plates as in Chapter 5.

[^3]H GABA Uptake and Autoradiography

Cultures were rinsed twice with Hanks/HEPES salts solution (Gibco-Biocult, or prepared to their formulation) and then incubated at 37 °C for 5-40 minutes in Hanks/HEPES containing 2 x 10^-7 M[^3]H GABA (4-amino-n- 2,3-^3 H -butyric acid, 54 Ci/m mole, Amersham) plus, where indicated, 10^-3 M β-alanine (Sigma) or 10^-3 M ACHC (cis-1,3-aminocyclohexanecarboxylic acid, supplied as a gift by Dr. N.G. Bowery, St. Thomas's Hospital Medical School). Incubation was terminated by three washes with Dulbecco's phosphate buffered saline (PBS), followed by fixation in 2.5% glutaraldehyde in PBS for 15 minutes at room temperature, three further PBS washes, one water wash, one 50% ethanol wash and two 95% ethanol washes. The cultures were air-dried and mounted cells upwards on microscope slides using UVInert mountant (BDH).

Ilford L-4 emulsion gel was melted at 43 °C in the dark and diluted 1 in 3 with water at the same temperature. The slides were dipped in diluted emulsion, excess emulsion wiped off, particularly from the back of the slide and allowed to dry for 30 minutes in the dark. Coated slides were stored in a light-tight box at -20 °C for 10 days. The silver grains were developed using Kodak Phen-X (8 minutes at room temperature), followed by 1% acetic acid (1 minute) and 30% sodium thiosulphate (10 minutes). The
autoradiograms were examined on a Zeiss Photomicroscope. However photography proved problematic in that lighting (bright-field) which gave a clear view of the grains did not reveal the unlabelled cells, while phase-contrast illumination obscured the grains. This would necessitate double exposures as used by Messer (1977). Counterstaining with a general cytoplasmic stain such as toluidine blue, as used by Lasher (1974), also tended to obscure the grains, particularly in monochrome photographs. It was found that differential interference contrast (Nomarski) optics gave a clear view of labelled and unlabelled cells and processes without the necessity for colour photography.

**Indirect Immunofluorescence**

Materials for this technique included antisera against four cell markers which were obtained as gifts from members of the Neuroimmunology Project, Dept. of Zoology, University College, London. These comprise: (a) Anti-GFA, a rabbit antiserum supplied by Dr. R. Pruss; (b) Anti-tetanus toxoid, a rabbit antiserum from Dr. R. Mirsky; (c) Anti-GalC (galactocerebroside), a rabbit antiserum from Professor M. Raff; (d) Anti-Thy 1, Dr. P. Lake supplied an allogeneic mouse antiserum to the allele Thy 1.1, raised in a mouse strain which bears Thy 1.2; rats all carry Thy 1.1. Tetanus toxin was given by Dr. R.O. Thompson of the Wellcome Laboratories, London. Antibody binding was visualised by the use of goat anti-rabbit Ig conjugated to rhodamine or fluorescein (GAR-R or GAR-F) or goat anti-mouse Ig conjugated to rhodamine or fluorescein (GAM-R or GAM-F); these conjugates were purchased from Nordic Labs Ltd.
All antisera were stored as frozen aliquots, or, for the aliquot in current use, at 4 °C with 0.02% sodium azide; they were centrifuged at 50,000g for 1 hour before use. Tetanus toxin as supplied was dialysed against PBS and stored frozen, a diluted aliquot for current use was kept at 4 °C with 0.02% sodium azide and similarly centrifuged before use. Safety precautions for the use of tetanus toxin require that all laboratory personnel be regularly immunised against tetanus, and that all solutions and equipment which have contacted toxin be autoclaved before disposal or washing, or be incinerated. Tetanus toxin was used at a final concentration of 10 μg/ml. Antisera were used at the following dilutions: (a) anti-GFA, 1 in 150 (b) anti-tetanus, 1 in 40 (c) anti-GalC, 1 in 25 (d) anti-Thy 1.1, 1 in 10 (e) Fluorescent conjugates, 1 in 40.

The procedure for indirect immunofluorescence described by Raff et al (1979) involves successive incubations at room temperature of cover-slip cultures with 40μl of diluted antiserum. This volume is the least which will cover the cover-slip, in order to minimise use of valuable antisera, but requires that the cover slip be incubated on a small "pedestal" to prevent surface tension drawing off the liquid. (The minimum volume which will cover a cover-slip placed in the bottom of a Multi-well is 200μl.) The cover-slips are washed between incubations by picking up each slip with forceps and dipping in a row of beakers containing rinse solution. This is not only a laborious procedure, but involves a high risk of breaking the fragile cover-slips. An improved procedure was devised involving
the design of a modified Multi-well plate which
allowed the rinsing and vacuum aspiration of up to 24 cultures
simultaneously without handling the cover-slips. Each well contained
a 1mm high neoprene 'pedestal' and had a 0.5mm hole drilled in the
design of a modified Multi-well plate which
bottom through to a lower chamber (actually a Falcon Multi-Well lid
adapted by the addition of a side outlet and glued to the bottom of
the Multi-Well plate) which could be evacuated by filter pump.

The protocol for indirect immunofluorescence was to remove
cover-slips from the culture well and place in a well of the special
dish. For surface labelling, they were washed twice with rinse
solution (Hanks/HEPES salts containing 5% Gibco normal lamb serum),
and then incubated for 30 mins at room temperature with 40μl of a
specific antiserum raised in rabbit or mouse diluted in MEM/HEPES
medium (Gibco) with 20% lamb serum. This was followed by 4 washes
with rinse solution, the second incubation with a fluorescent
conjugate raised in goat against the Ig of rabbit or mouse as
appropriate, 4 more washes, then fixation for 10 mins at -20 °C in
5% acid alcohol (acetic acid in ethanol). Tetanus labelling
involved three incubations, the first with toxin, then anti-tetanus,
and finally conjugate. Labelling with anti-GFA, an internal marker
required fixation in acid alcohol before the antibody incubations.
Cover slips were mounted face down in glycerol and sealed with UV
Inert mountant or nail polish. They were observed on a Zeiss
Photomicroscope equipped with epifluorescence, including rhodamine and
fluorescein filters, and a 63x Planapochromat phase contrast lens,
and photographed using Kodak ASA400 colour slide or monochrome film.
In order to confirm the specificity of labelling of different cell types it was necessary to double label a single culture with more than one antibody. If the two specific antisera to be used were raised in different species, then they were followed by a fluorescein-linked conjugate against the Ig of one species, and a rhodamine-linked conjugate against the other. The two specific antibodies could be mixed, and then followed by a mix of conjugates (unless double labelling with a surface and an internal marker, where one had to be before and one after fixation). Double labelling was also possible if both specific antisera were raised in the same species. In this case however, the labels were distinguished by ordering the incubations so that the second specific antiserum was applied after one of the conjugates. The final incubation with the other conjugate labelled both specific antisera. Thus the procedure for double labelling with anti-GFA and tetanus toxin, in Figures 6.8-6.10, was to first incubate with tetanus toxin, followed by rabbit anti-tetanus, then goat anti-rabbit Ig-rhodamine, followed by fixation, then rabbit anti-GFA, and finally goat anti rabbit Ig-fluorescein. This resulted in the first specific antiserum (anti-tetanus) being labelled by rhodamine and fluorescein, while the second specific antiserum (anti-GFA) was labelled by fluorescein alone.

RESULTS

Neuronal-specific and glial-specific GABA uptake

Figure 6.3a shows the uptake pattern of $[^3H]GABA$ into a cerebellar
TCS culture revealed by autoradiography. Several features are apparent. The numerous small rounded cell bodies and processes of the neurons identified in Chapter 5 as granule cells on the basis of their morphology, migratory behaviour and tendency to aggregate are completely unlabelled by $^3\text{H}GABA$. There is moderate labelling of most or all of the flattened 'background' cells which tend to divide if not inhibited by FUDR, note that these include some more rounded, process-bearing cells (arrowed). Finally, there is heavy labelling of a minority population of cells which are of quite variable appearance. Some with many short branching processes resemble astrocytes (as also suggested by Lasher, 1974) while others with fewer processes that may extend for long distances are more typically neuron-like. The cell bodies also vary in size, being generally 15-25μm in diameter, but some are as small as granule cells (8μm).

Uptake into cerebellar cultures of $^3\text{H}GABA$ (0.2μM) was also assessed in the presence of inhibitory (competitive) concentrations (1 mM) of GABA analogues which are selective for the high-affinity GABA transport sites of neurons (ACHC) or of glia (β-alanine). Figure 6.1b shows that in the presence of ACHC, the uptake into background, probable glial, cells is unaffected, including those bearing processes. However the heavy labelling of some process-bearing cells seen in Figure 6.1a is no longer apparent. In the converse situation of inhibition by β-alanine, which is selective for glial uptake, (Figure 6.1c) a substantial reduction in uptake by the flattened background cells can be seen, while there is no effect on uptake by the heavily-labelled process-bearing cells including those of 'astrocytic' morphology. Figure 6.1d shows that in the presence of both competitors
Figure 6.1: Autoradiographs showing uptake of $[^3\text{H}]\text{GABA}$ ($2 \times 10^{-7}\text{M}$, 40 min.) into cultures of 7-day rat cerebellum TCS, 4 DIV. 

a: $[^3\text{H}]\text{GABA}$ in the absence of inhibitors, note that the aggregating granule cells are unlabelled, that the moderately labelled background cells include some process-bearing cells (arrow), and that the heavily-labelled process-bearing cells are of a wide range of size and shape. 

b: $[^3\text{H}]\text{GABA}$ in the presence of ACHC ($10^{-5}\text{M}$), a competitive inhibitor of neuronal high-affinity GABA uptake, note that uptake into background cells is unaffected while uptake into the heavily labelled cells seen in a is inhibited.
Figure 6.1 (cont): c: $[^3H]$GABA in the presence of β-alanine ($10^{-3}$M), a competitive inhibitor of glial high-affinity uptake, note that uptake into heavily-labelled process-bearing cells is unaffected, while uptake into the background cells is markedly reduced. d: $[^3H]$GABA in the presence of both ACHC ($10^{-3}$M) and β-alanine ($10^{-3}$M). Uptake into all cell types is inhibited. Scale bar 50μm.
uptake into all cells is inhibited. These results indicate that neuronal-specific and glial-specific uptake can be distinguished in vitro using these competitors as already shown in vivo.

These experiments were also performed in parallel on cultures prepared from olfactory bulb. Figure 6.2a shows the pattern of uptake of \([^3\text{H}]\text{GABA (0.2}\mu\text{M})\) into a rat olfactory bulb culture, 4DIV. The contrast with cerebellar cultures is immediately apparent, the great majority of the small (<10\mu m) rounded process-bearing cells are heavily labelled by \([^3\text{H}]\text{GABA, although it should be noted that a few small cells of such 'neuronal' morphology remain unlabelled. There is moderate labelling of the flattened background cells. In the presence of ACHC (1mM), the neuronal uptake competitor, labelling of the neuronal cell bodies and their processes is greatly inhibited (Figure 6.2b), while uptake into background cells is not affected. \(\beta\)-alanine (1mM), the glial-specific competitor, markedly reduces uptake into these flattened cells, but does not affect uptake into the small neurons (Figure 6.2c). These clear-cut results support the interpretation placed on the competitor results in the cerebellum cultures that ACHC and \(\beta\)-alanine are, in this system, distinguishing neuronal-specific and glial-specific GABA uptake.

In order to establish that specific GABA uptake is Na\(^+\)-dependent in vitro as it is in vivo, uptake experiments were performed in a Na\(^+\)-free medium. Figure 6.2d shows that uptake of \([^3\text{H}]\text{GABA (0.2}\mu\text{M})\) into an olfactory bulb culture does not occur in the absence of Na\(^+\).
Figure 6.2: Autoradiographs showing uptake of $[^{3}H]_{\text{GABA}}$ (2 x $10^{-7}$ M, 40 min.) into cell cultures of 5-day rat olfactory bulb, 4 DIV. a: $[^{3}H]_{\text{GABA}}$ in the absence of inhibitors, note that in olfactory bulb cultures most small neurons show GABA uptake, although some small cells of neuronal morphology are unlabelled. b: $[^{3}H]_{\text{GABA}}$ in the presence of ACHC ($10^{-3}$ M), the neuronal uptake inhibitor, uptake into small neurons is clearly inhibited while labelling of background cells is unaffected.
Figure 6.2 (cont): 

$\text{c: } ^{3}\text{H}\text{GABA in the presence of } \beta\text{-alanine (10}^{-3}\text{M)},$  
the glial uptake inhibitor, uptake into neuronal cell bodies and 
processes is unaffected while background uptake is reduced. Note the 
two large unlabelled process-bearing cells, these may be glial, but 
resemble neurons; olfactory bulb contains large excitatory neurons 
(mitral cells). 

$\text{d: } ^{3}\text{H}\text{GABA in the absence of inhibitors, but in a}$ 
$\text{Na}^-\text{free medium. Scale bar 50} \mu\text{m. Based on choline chloride. The addition}$ 
of $15\text{mM NaCl to this incubation medium restored uptake (not shown)}$ 
$\text{Scale bar 50} \mu\text{m.}$
Table 6.1

Distribution of $[^3\text{H}]\text{GABA}$ labelling in Cerebellar and Olfactory Bulb Cultures (% of total cells ± S.E.M.)

<table>
<thead>
<tr>
<th></th>
<th>Unlabelled Neurons</th>
<th>$[^3\text{H}]\text{GABA}$ labelled Neurons &lt;10μm</th>
<th>$[^3\text{H}]\text{GABA}$ labelled Neurons &gt;10μm</th>
<th>$[^3\text{H}]\text{GABA}$ labelled Non-neuronal cells (&gt;90% Astrocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebellar Cultures:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>77.5 ± 1.3</td>
<td>2.2 ± 0.2</td>
<td>5.4 ± 0.3</td>
<td>14.8 ± 1.1</td>
</tr>
<tr>
<td>Olfactory Bulb Cultures:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.2 ± 1.8</td>
<td>51.9 ± 2.6</td>
<td>1.9 ± 0.2</td>
<td>31.0 ± 3.9</td>
</tr>
</tbody>
</table>

In each tissue, data from two experiments with counts of >1000 cells.
Thus examination of autoradiograms of $[^3]H$GABA uptake into cultures of cerebellum and olfactory bulb allows several classes of cells to be distinguished. The proportions of cells seen in the selected photographs shown are confirmed when counts across many randomly selected fields of different cultures in different experiments are taken. Table 6.1 shows that the small unlabelled putative granule neurons are over 75% of cerebellar cultures. There are 6% of the larger neurons which are labelled, and therefore GABAergic if the principle established in vivo (Schon and Iversen, 1974) holds true in vitro. Over 50% of the cells in olfactory bulb cultures are small neurons which accumulate GABA.

**Immunological markers for cell type**

Internal labelling of cells in cerebellar cultures by anti-GFA results in clear marking of filaments inside the majority of flattened background cells. (Figure 6.3). There is no specific marking of the putative granule neurons, or of two minor cell populations (see below). The astrocytes revealed by this method are of a wide range of appearance, including large flattened cells and cells with a more defined cell body bearing processes. This provides effective confirmation that the majority of background cells, which were found above to be showing glial-specific GABA uptake, are indeed astrocytes.

Surface binding of tetanus toxin is found on the putative granule neurons and their processes (Figure 6.4). Immunofluorescent
labelling of tetanus toxin is particularly effective in revealing
the great extent of the neuritic net formed in these cultures. Large
bundles of fibres are labelled as are a multitude of fine individual
processes. Figure 6.4 demonstrates that flattened cells do not
label with tetanus, in particular an unlabelled flattened cell
(presumptive astrocyte) can be seen with a network of labelled
neuronal processes running over its surface. Occasionally, larger
tetanus-positive neurons can be seen (Figure 6.5). The small
cell bodies and processes of olfactory bulb neurons are also found
to be tetanus-positive (Figure 6.5).

A small proportion of cells in cerebellar cultures (<0.5%) are
labelled by anti-galactocerebroside, however these few are brightly
positive and have the same characteristic morphology as the
oligodendrocytes described by Raff et al (1978). Figure 6.6
illustrates the defined cell body and extensive system of processes
often displayed by these cells. It is extremely doubtful whether
these cells could have been distinguished from neurons, or even
recognised as a discrete population on morphological grounds without
the assistance of a specific marker.

Labelling by anti-Thy 1.1 was also examined in cerebellar
cultures from one week postnatal rats. Up to seven days in vitro,
the only Thy 1 labelling was found on the surface of a minor
population of flattened cells, which were not morphologically
distinguishable from the more flattened astrocytes (Figure 6.7).
Double labelling showed that there was no overlap between GFA-positive
Figure 6.3: Anti-GFA labelling of astrocytes viewed with phase contrast (left) fluorescence (right) optics. Top: R7-TCS, 3 DIV. Note the varying morphology of the GFA-positive cells. Scale bar 50μm.
Bottom: R7-TCS, 4 DIV. This lower power view shows the range of morphology of astrocytes in culture from flattened cells to cells showing extensive process outgrowth. Scale bar 50 μm. Cultures were fixed in acid alcohol, then incubated with rabbit anti-GFAP, followed by GAR-R. The faint fluorescence of the rounded granule cell perikarya is non-specific autofluorescence, also present in controls.
Figure 6.4: Tetanus toxin labelling of neurons in 7 day rat cerebellar culture, 11 DIV. Top: phase contrast. Bottom: fluorescence optics. Note that brightly labelled granule neuron processes can be seen passing over the surface of unlabelled probable astrocytes. Cultures were incubated with tetanus toxin, followed by rabbit anti-tetanus, then GAR-R. Scale bar 50µm.
Figure 6.5: Tetanus toxin labelling viewed with phase contrast (left) and fluorescence (right) optics. Top: R7-TCS, 9 DIV. A larger neuron present in a culture where glial overgrowth has been permitted and granule neurons have therefore died off, see also Fig 6.10. Scale bar 50 μm. Bottom: Olfactory bulb culture, 9 DIV. The small cell bodies and processes of neurons present in olfactory bulb cultures are also tetanus positive, compare Fig. 6.2. Scale bar 50 μm.
Figure 6.6: Labelling of oligodendrocytes in cerebellar cultures, 4 DIV by anti-galactocerebroside viewed by phase contrast (left) and fluorescence (right) optics. Note the distinctive process-bearing morphology of these cells when labelled, and the lack of any distinguishing characteristics in phase contrast. Cultures were incubated with rabbit anti-GaLC followed by GAR-R. Scale bar 50μm.
Figure 6.7: Anti-Thy 1 labelling of fibroblasts. Top: Phase contrast (left) and rhodamine/flourescein double exposure (right) of R7-TCS culture, 11 DIV. double labelled with anti-Thy 1 and tetanus toxin. Note that the large flattened Thy 1-positive cell is difficult to see in phase contrast, and that other flattened cells, probably astrocytes, can be seen in phase but are not labelled by anti-Thy 1. The culture was incubated with tetanus toxin, followed by rabbit anti-tetanus and mouse anti-Thy 1, and then GAR-F and GAM-R. Scale bar 50μm. Bottom: Rhodamine/flourescein double exposure of R7-TCS culture, 6 DIV double labelled with anti Thy 1 (rhodamine-linked) and anti-GFAP (fluorescein-linked). There is no overlap between the two populations of flattened cells defined by these markers. The culture was incubated with mouse anti-Thy 1, then GAM-R, followed by fixation in acid/alcohol, then rabbit anti-GFA, and finally GAR-F. Scale bar 50μm.
astrocytes and these Thy 1-positive cells. This is in accord with the results of Mirsky and Thompson (1975), and Raff et al (1979), who argue that these cells are fibroblasts. They will be designated here as fibroblasts although the possibility cannot be excluded that Thy 1-positive, GFA-negative flattened cells are a mixed population including other non-neuronal cells such as endothelial cells. In any case, they represent a small minority (<1%) of the cells in cerebellar cultures.

During the second week in vitro the granule neurons begin to develop Thy 1 on their surfaces, as has been reported elsewhere (Currie et al, 1977). It seems probable that Thy 1 appears on neurons as a function of development (see Raff et al, 1979) and therefore the late-developing granule neurons are delayed in their expression of Thy 1. This accords with the time course of Thy 1 development in rat brain which involves an increase from very low levels at birth to virtually adult levels after three weeks (Douglas, 1972). It has also been reported that Thy 1 develops on some astrocytes after about 2 weeks in vitro (Pruss, 1979), but GFA labelling continues to distinguish these from fibroblasts.

The specificity of these markers in this system was further confirmed by double labelling experiments. Figure 6.8 shows a cerebellum culture, 3 days in vitro, double labelled with tetanus toxin and anti-GFA as described in Methods. The extensive network of neuronal processes is clearly revealed as are the underlying astrocytes. Figure 6.9 shows a similar culture at 11 days in vitro,
Figure 6.8: Tetanus toxin/anti-GFA double labelling, R7-TCS, 3 DIV. Procedure described in Methods section. This rhodamine/fluorescein optics double exposure shows the fluorescein-linked anti-GFA labelling as green and the rhodamine and fluorescein-linked tetanus labelling as yellow (red + green = yellow on colour film). The dense net of neuronal processes formed during the first three days in culture is apparent. Scale bar 50μm.
Figure 6.9: Tetanus toxin/anti-GFA double labelling, R7-TCS, 14 DIV. Labelling as in Fig. 6.8. The effects of the fasciculation of processes during the second week in vitro are seen by comparing this with Fig. 6.8. Note also the tendency for processes to spread out over the surface of glial cells while remaining in bundles over the polylysine surface. In the lower right can be seen a neuronal process following a glial process for some distance. Scale bar 50μm.
Figure 6.10: Tetanus toxin/anti-GFA double labelling, R7-TCS, 14 DIV, grown with 5mM K* and no FUDR. Labelling as in Figure 6.8. Two surviving neurons and their processes can be seen in this culture grown under conditions which favour glia. Scale bar 50um.
Figure 6.11: A large neuron in R7-TCS culture, grown in 5mM K+ 11 DIV. Top: Tetanus toxin labelling showing extensive bipolar neuritic outgrowth. Bottom: Phase contrast (left) and tetanus toxin/anti-GFA double labelling (right, Labelling as in Figure 6.8) of the same cell at high power. Note the field has shifted slightly between exposures giving an imperfect double exposure which however serves to illustrate the separate red and green images which make up the yellow image seen in Figs. 6.8-6.11. The 63 x Planapochromat lens used here has a narrow depth of field, hence the labelling on the top of the cell and the GFA labelling of background astrocytes is out of focus. Scale bar 50μm.
the migration of cell bodies and fasciculation of processes is particularly clearly illustrated here. Figure 6.10 shows similar labelling of a culture seeded at the same density which has been grown for 14 days in vitro without mitotic inhibitor or high K⁺ i.e. favouring glia over neurons. The monolayer of astrocytes is clearly labelled and, in this selected field, two surviving neurons and their processes are shown by tetanus labelling. Large neurons can occasionally be distinguished by tetanus labelling in cultures grown under normal or, as in Figure 6.10, low K⁺ conditions. The large neuron shown in Figure 6.11 is 20-30μm in soma diameter and, by its pattern of neurite outgrowth, and its appearance in phase contrast highly reminiscent of a Purkinje cell. However without a specific marker for Purkinje cells, it would be inappropriate to make such an attribution on the basis of morphology alone. The cell could also be a Golgi neuron or large basket cell. Such cells are extremely rare in cultures from this age of animal (but see below).

It was found that all cells in the culture labelled with one of these four markers. There is therefore no substantial class of cells which does not fall into one of the four categories: neurons, astrocytes, oligodendrocytes, and 'fibroblasts'. Table 6.2 shows the proportions in which these classes occur in culture. The possibility remains that a type of cell is present in very low proportion (<<0.5%) which is negative for all four markers and has remained unnoticed in phase contrast, as oligodendrocytes probably would have without a specific marker.
Table 6.2  PROPORTIONS OF IDENTIFIED CELL CLASSES IN CEREBELLAR CULTURES

<table>
<thead>
<tr>
<th>Phase-contrast appearance</th>
<th>Immunolabelling</th>
<th>Proportion of R7-TCS culture</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>8µm rounded cell body bearing few, long processes; cell bodies aggregate and processes fasciculate</td>
<td>None</td>
<td>(-) 75-85%</td>
<td>Granule neurons</td>
</tr>
<tr>
<td>Flattened cells</td>
<td>Glial-specific</td>
<td>7-15%</td>
<td>Astrocytes</td>
</tr>
<tr>
<td>&gt;15µm defined cell body with processes</td>
<td>Glial-specific</td>
<td>1-2%</td>
<td></td>
</tr>
<tr>
<td>10-25µm defined cell body with processes</td>
<td>Neuron-specific</td>
<td>4-6%</td>
<td>Inhibitory interneurons (stellate and basket cells)</td>
</tr>
<tr>
<td>20-30µm defined cell body with processes</td>
<td>Neuron-specific</td>
<td>0 (Only present in embryonic cultures)</td>
<td>Inhibitory neurons (Purkinje cells?)</td>
</tr>
<tr>
<td>10-15µm defined cell body with processes</td>
<td>Not known</td>
<td>0.1-0.3%</td>
<td>Oligodendrocytes</td>
</tr>
<tr>
<td>Flattened cells</td>
<td>Not known</td>
<td>0.2-1.0%</td>
<td>&quot;Fibroblasts&quot;</td>
</tr>
</tbody>
</table>
Embryonic cerebellar cultures

Thy 1 labelling has led to the recognition of a class of Thy 1-positive, tetanus-positive neurons in cerebellar cultures prepared from 17-19 day embryos which are missing from cultures prepared as above, from one week postnatal rats. This work was performed in collaboration with Dr. K. Fields of University College, London (now Albert Einstein College, New York) and has been reported in preliminary form elsewhere (Currie et al., 1977). Figure 6.12 illustrates these cells, which in phase contrast are always large (>20μm) with a clearly visible nuclear membrane and a single prominent nucleolus. The generally ovoid cell body bears one to six processes which can extend for great distances (>1mm) through the culture. There are also other large tetanus-positive neurons in the culture which are Thy 1 negative, as are the large putative inhibitory neurons found in cultures of one week cerebellum.

GABA autoradiography on cultures of embryonic cerebellum reveals a picture (Figure 6.13) of overall similarity to the cultures of older animals, including β-alanine sensitive labelling of glia, unlabelled (which are not present at this age in vivo, but could develop from precursor granule neurons), and AChC-sensitive labelling of some neurons. However these last are in a higher proportion and, although exhibiting a wide range of appearances, include a population of large neurons which are morphologically similar to the Thy 1 positive population. It has not proven possible to double label a culture with [3H]GABA and immunofluorescence due to interference of the glutaraldehyde required for GABA fixation. However counts and size measurements
Figure 6.12: Anti-Thy 1 labelling of large neurons in cultures of embryonic rat cerebellum, 7 DIV, viewed by phase contrast (left) and fluorescence (right) optics. Using the 63x Planapochromat lens it is difficult to focus the whole of these large cells, but note the large nucleolus and nuclear membrane. Scale bar 50μm.
Figure 6.13: Uptake of $[^3\text{H}]$GABA (2 x $10^{-7}$M) into cultures of embryonic rat cerebellum, 7 DIV, revealed by autoradiography. Top: in the presence of $10^{-3}$M $\beta$-alanine. Bottom: in the presence of $10^{-3}$M ACHC. Note the occasional very large neuron which is heavily labelled, this uptake shows neuronal specificity in that it is inhibited by ACHC and not by $\beta$-alanine. Scale bar 50μm.
of cells labelled by the two techniques reveals a correspondence of a proportion (generally 10-20%) of cells with cell body of 20\(\mu\)m x 30\(\mu\)m (Fields, Currie, Dutton in preparation). It thus appears that a subset of the putative GABAergic neurons found in embryonic cerebellum cultures are large oval Thy 1 positive cells of a characteristic morphology. These cells are present in much reduced numbers in cultures of newborn rat (Currie et al, 1977) and are almost entirely missing (but see Figure 6.11) from the cultures of one week postnatal animals described in detail above.

DISCUSSION

Using a combination of cell type markers on cerebellar cultures it has proven possible to define several classes of cell by a range of criteria. These are summarised in Table 6.2. The most numerous class of cell were tentatively identified in the previous chapter as granule neurons on the basis of size, numbers, pattern of neurite outgrowth and migratory behaviour. The results described here, in particular their lack of GABA uptake, strongly support this conclusion. There is now no other tenable possibility for the identity of these cells if they are, as they appear by all criteria, a homogeneous population. The migration of granule cells into aggregates is shown by the autoradiography results to be quite specific. The inhibitory neurons present in the cultures show no tendency to migrate into granule neuron aggregates, or to form their own aggregates. Inhibitory neurons are sometimes seen within granule neuron aggregates but at a frequency quite consistent with occasional random inclusion.
The major population of background, flattened cells have been confirmed as astrocytes by the GABA uptake work, and, most certainly, by labelling with anti-GFA. These are the population of cells which will divide and take over the culture unless prevented by mitotic inhibitor. There is notable morphological similarity between the small proportion of astrocytes which have a defined cell body with multiple processes and some of the cells which show neuronal-specific GABA uptake. The biochemical criterion is likely to be far more reliable than morphology, and this serves to illustrate the pitfalls in attributions of cell type on the basis of phase contrast unsupported by more specific markers. The probable presence of oligodendrocytes and fibroblasts has been confirmed, but their numbers have been shown to be small.

The cerebellum, as described in Chapter 1, contains a restricted number of cell populations including just five neuronal types and the usual CNS non-neuronal cell types. Oligodendrocytes, astrocytes, fibroblasts, and granule neurons have been identified and quantified. There remain Purkinje neurons and the three classes of inhibitory neurons: stellate, basket and Golgi cells. All of these may be expected to show GABA uptake in vitro (see Introduction) and so must together account for the GABA-labelled neurons observed in cerebellar cultures. Stellate and basket cells are present as dividing precursors or are immediately post-mitotic at one week postnatal in the rat (Altman, 1972a). They may therefore be expected to be better able to survive the cell isolation procedure than the earlier developing and in any case less numerous Golgi and Purkinje neurons. From this
argument, and from their size (10-20μm) it is therefore likely that all or the great preponderance of the GABA labelled neurons seen in cultures of one week cerebellum are stellate and basket cells. The presence of a small proportion of Golgi neurons cannot be ruled out. As to Purkinje cells, it was observed in the work described in the previous Chapter that separated Purkinje neurons did not survive in culture, and this was believed to also apply to unseparated Purkinje neurons in cultured TCS. A few examples of probable surviving Purkinje cells were shown. It is also possible, using markers to find occasional cells which strongly resemble Purkinje neurons (Figure 6.11), however these are a rarity. All the evidence points to a lack of survival of Purkinje neurons in any number.

However a class of large GABA-positive, Thy 1-positive neurons was found in cultures of embryonic cerebellum which was absent in cultures of older animals. Although the evidence is at best circumstantial, these cells may be Purkinje neurons. As already described, Thy 1 seems to appear on the surface of neurons as they mature. Cultures of one week postnatal cerebellum contain only neurons which develop Thy 1 during the second week in vitro. Yet embryonic cultures contain characteristic large neurons which exhibit Thy 1 during the first few days in vitro. Either these cells develop Thy 1 in culture more slowly when isolated from a more mature cerebellum, or they fail to survive in culture due to the damage inflicted on an early-developing neuron by cell dissociation. The second possibility seems far more likely, that the Thy 1-positive GABA-positive neurons seen in cultures of embryonic cerebellum are an early-developing class of large GABAergic
neurons which do not survive in cultures of older animals. They are thus most probably Purkinje neurons, although Golgi neurons could also fulfil this description, but these are in smaller numbers than Purkinje neurons. The large neurons seen in culture are mature generally multipolar whereas Purkinje cells are bipolar in vivo. However it need not be expected that cells will reproduce their exact outgrowth pattern in the absence of cues such as the normal cortical layering. In fact Purkinje cells can be multipolar in vivo when growing in a disrupted environment such as the irradiated agranular cerebellum (Berry and Bradley, 1976) or the reeler mutant (Caviness and Rakic, 1978).

Cultures of the olfactory bulb are much less well-defined than cerebellar cultures. However the olfactory bulb is also an area containing a restricted number of known cell types, and would be susceptible to the same sort of analysis. Clearly olfactory bulb cultures are highly enriched in GABAergic neurons and may be an excellent system for biochemical studies of such cells.
In the preceding chapters has been described the isolation and separation of cells from the developing rat cerebellum, their survival and development in primary monolayer culture, and the definition of the composition of such cultures using several markers for cell type. Several possible future improvements to the details of this system have been mentioned, for instance the possibility that separated Purkinje cells (Chapter 3) will survive if prepared from younger animals, the results of Thy 1 labelling embryonic cultures (Chapter 6) support this suggestion.

It remains to set some broad directions for future progress using a system of this type. The importance for the biochemist of accurate knowledge of cellular composition was discussed in Chapter 4.
By starting with a brain area containing a limited range of cell
types of known properties, and using a range of cell markers, it
has been possible to produce defined cell cultures of quantified
composition. Three inter-dependent directions of work with these
cultures will be mentioned. Firstly, the cultures can be further
developed by enriching for particular cell types. As described
in Chapter 5, cerebellar cultures are over 80% in granule neurons; glial
cultures (>95% astrocytes) can be prepared using a different medium
(Chapter 6). It may be possible, for instance, to select against
inhibitory neurons using kainic acid as described in vivo (Herndon
and Coyle, 1977). This would result in bulk cultures of a single
neuron type, granule cells. Secondly, the cultures may be used for
functional studies of neuronal development. The timetable of
development of action potential Na⁺ channels in these cultures has
been studied (Beale et al, 1980). Stimulated, Ca²⁺-dependent release
of GABA has been monitored (Pearce, Currie, Beale and Dutton, submitted),
and this work can be extended to the study of release of other
candidate neurotransmitters. The excitatory transmitter of the
granule cells is not known, although there is some circumstantial
evidence for glutamate (e.g. Young et al, 1974) and granule cell-
enriched cultures may be ideal for testing this and other candidates.

These two areas of effort may be regarded as short or medium-term.
The long-term direction which this author sees as most promising is
to link the culture system with modern immunological techniques. It
is now possible with the lymphocyte hybridoma technique (Kohler and
Milstein, 1975) to prepare large quantities of pure monospecific
antibodies against unknown unpurified antigens. However the considerable technical problems are reduced if the inoculum is as simple as possible. Enriched monolayer cultures provide a simplified system which are advantageous in this respect, and which can also be used to assay hybridoma supernatants (Schneider and Eisenbarth, 1979).

The results of such a study could be highly important. It should be possible to identify new markers for cell types, perhaps distinguishing individual neuronal types in culture. This has the immediate practical advantages of more accurate definition of culture composition, and of culture purification by positive separation of cells by cell-sorting or affinity methods (Au and Varon, 1979), and negative selection by complement-mediated cytotoxicity. All the 'tricks' of selective media may be foregone in favour of selection of a chosen cell type from a mixed cell suspension using specific antibodies. This would then allow the creation of pure cultures in which the effects of intrinsic and extrinsic factors on cellular development (Chapter 1) could be studied down to the most detailed biochemical level. Monospecific antibodies against unknown cell-specific antigens also provide the first really promising means to look for the 'recognition molecules' (Chapter 1) if such exist. An antibody is a ligand which can be used to determine the distribution of the antigen, its timetable of development, to interfere with and thus study its function, and finally to purify the antigen molecule (Sunderland et al, 1979).
The importance of immunological techniques for the future development of neurobiology is assured. By linking these techniques with defined cell cultures, each amplifies the other. Cultures are an excellent simple system for the preparation and assay of new monoclonal antibodies which can then be used to improve the culture system. In the context of such possibilities the work described in the preceding chapters can be seen to represent only a few tiny steps but perhaps in the right direction.
REFERENCES


ALTMAN J. and ANDERSON W.J. (1973) Experimental reorganization of the cerebellar cortex. II. Effects of elimination of most microneurons with prolonged X-irradiation started at four days. J. Comp. Neurol. 149, 123-152.


BUNGE M.B., BUNGE R.P. and PETERSON E.R. (1967) The onset of synapse formation in spinal cord cultures as studied by electron microscopy Brain Res. 6, 728-749.


BURRY R.W. and Lasher R.S. (1978a) A quantitative electron microscopic study of synapse formation in dispersed cell cultures of rat cerebellum, stained either by Os-UL or by E-PTA. Brain Res. 147, 1-15.


GREENE L. and TISCHLER A.S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to NGF. PNAS 73, 2424-2428.


HÖSLE L., HÖSLE E. and ANDRES P.F. (1973) Nervous tissue cultures - a model to study action and uptake of putative neurotransmitters such as amino acids. Brain Res. 62, 597-602.


LANDIS S.C. (1976) Rat sympathetic neurons and cardiac myocytes developing in microcultures: correlation of the fine structure of endings with neurotransmitter function in single neurons PNAS 73, 4220-4224.


MCCARTHY K.D. and PARTLOW L.M. (1976b) Neuronal stimulation of \(^3\)H\)thymidine incorporation by primary cultures of highly purified non-neuronal cells. 114, 415-426.


NAKAI J. (1960) Studies on the mechanism determining the course of nerve fibers in tissue culture. II. The mechanism of fasciculation Z. Zellforsch. 52, 427-499.

NAKAI J. and KAWASAKI Y. (1959) Studies on the mechanism determining the course of nerve fibers in tissue culture. I. The reaction of the growth cone to various obstructions. Z. Zellforsch. 51, 108-122.


RAKIC P. and SIDMAN R.L. (1973a) Weaver mutant mouse cerebellum: defective neuronal migration secondary to specific abnormality of Bergmann glia. PNAS 70, 240-244.


SATO G. (1973) Tissue culture of the nervous system. Plenum, N.Y.


SENSENBRENNER M., BOOHER J. and MANDEL P. (1971) Cultivation and growth of dissociated neurons from chick embryo cerebral cortex in the presence of different substrates. Z. Zellforsch. 117, 559-569.


SOTELO C. (1975) Anatomical, physiological and biochemical studies of cerebellum from mutant mice. II. Morphological study of cerebellar cortical neurons and circuits in the weaver mouse. Brain Res. 94, 19-44.

SOTELO C. and CHANGEUX J.P. (1974) Trans-synaptic degeneration "en cascade" in the cerebellar cortex of staggerer mutant mice; Brain Res. 67, 519-526.


STEINER G. and SCHONBAUM E. (1972) Immunosympathectomy, eds. Elsevier, N.Y.


VARON S., RAIBORN C.W. Jr. and NORR S. (1974b) Association of antibody to nerve growth factor with ganglionic non-neurons (glia) and consequent interference with their neuron-supportive action. Exp. cell Res. 86, 247-256.


